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## **Alzheimer's Disease Secretase, APP Substrates Therefor, and Uses Therefor**

5 The present application is a continuation of United States Application  
Serial No. 09/416,901, filed October 13, 1999 which claims priority benefit of United  
States Provisional Patent Application No. 60/155,493, filed September 23, 1999. The  
present application also claims priority benefit as a continuation-in-part of United  
States Patent Application Serial No. 09/404,133 and PCT/US99/20881, both filed  
September 23, 1999, both of which in turn claim priority benefit of United States  
Provisional Patent Application No. 60/101,594, filed September 24, 1998. All of  
10 these priority applications are hereby incorporated by reference in their entirety.

### **FIELD OF THE INVENTION**

The present invention relates to Alzheimer's Disease, amyloid protein  
precursor, amyloid beta peptide, and human aspartyl proteases, as well as a method for  
15 the identification of agents that modulate the activity of these polypeptides and  
thereby are candidates to modulate the progression of Alzheimer's disease.

### **BACKGROUND OF THE INVENTION**

Alzheimer's disease (AD) causes progressive dementia with consequent  
20 formation of amyloid plaques, neurofibrillary tangles, gliosis and neuronal loss. The  
disease occurs in both genetic and sporadic forms whose clinical course and  
pathological features are quite similar. Three genes have been discovered to date  
which, when mutated, cause an autosomal dominant form of Alzheimer's disease.  
These encode the amyloid protein precursor (APP) and two related proteins,  
25 presenilin-1 (PS1) and presenilin-2 (PS2), which, as their names suggest, are  
structurally and functionally related. Mutations in any of the three proteins have been  
observed to enhance proteolytic processing of APP via an intracellular pathway that  
produces amyloid beta peptide (A $\beta$  peptide, or sometimes here as Abeta), a 40-42  
amino acid long peptide that is the primary component of amyloid plaque in AD.

Dysregulation of intracellular pathways for proteolytic processing may be central to the pathophysiology of AD. In the case of plaque formation, mutations in APP, PS1 or PS2 consistently alter the proteolytic processing of APP so as to enhance formation of A $\beta$  1-42, a form of the A $\beta$  peptide which seems to be particularly amyloidogenic, and thus very important in AD. Different forms of APP range in size from 695-770 amino acids, localize to the cell surface, and have a single C-terminal transmembrane domain. Examples of specific isotypes of APP which are currently known to exist in humans are the 695-amino acid polypeptide described by Kang *et al.* (1987), *Nature* 325: 733-736 which is designated as the "normal" APP; the 751 amino acid polypeptide described by Ponte *et al.* (1988), *Nature* 331: 525-527 (1988) and Tanzi *et al.* (1988), *Nature* 331: 528-530; and the 770 amino acid polypeptide described by Kitaguchi *et al.* (1988), *Nature* 331: 530-532. The A $\beta$  peptide is derived from a region of APP adjacent to and containing a portion of the transmembrane domain. Normally, processing of APP at the  $\alpha$ -secretase site cleaves the midregion of the A $\beta$  sequence adjacent to the membrane and releases the soluble, extracellular domain of APP from the cell surface. This  $\alpha$ -secretase APP processing creates soluble APP-  $\alpha$ , which is normal and not thought to contribute to AD. Pathological processing of APP at the  $\beta$ - and  $\gamma$ -secretase sites, which are located N-terminal and C-terminal to the  $\alpha$ -secretase site, respectively, produces a very different result than processing at the  $\alpha$  site. Sequential processing at the  $\beta$ - and  $\gamma$ -secretase sites releases the A $\beta$  peptide, a peptide possibly very important in AD pathogenesis. Processing at the  $\beta$ - and  $\gamma$ -secretase sites can occur in both the endoplasmic reticulum (in neurons) and in the endosomal/lysosomal pathway after reinternalization of cell surface APP (in all cells). Despite intense efforts, for 10 years or more, to identify the enzymes responsible for processing APP at the  $\beta$  and  $\gamma$  sites, to produce the A $\beta$  peptide, those proteases remained unknown until this disclosure.

## SUMMARY OF THE INVENTION

Here, for the first time, we report the identification and characterization of the  
30  $\beta$  secretase enzyme, termed Aspartyl Protease 2 (Asp2). We disclose some known

and some novel human aspartic proteases that can act as  $\beta$ -secretase proteases and, for the first time, we explain the role these proteases have in AD. We describe regions in the proteases critical for their unique function and for the first time characterize their substrate. This is the first description of expressed isolated purified active protein of this type, assays that use the protein, in addition to the identification and creation of useful cell lines and inhibitors.

Here we disclose a number of variants of the Asp2 gene and peptide.

In one aspect, the invention provides any isolated or purified nucleic acid polynucleotide that codes for a protease capable of cleaving the beta ( $\beta$ ) secretase cleavage site of APP that contains two or more sets of special nucleic acids, where the special nucleic acids are separated by nucleic acids that code for about 100 to 300 amino acid positions, where the amino acids in those positions may be any amino acids, where the first set of special nucleic acids consists of the nucleic acids that code for the peptide DTG, where the first nucleic acid of the first special set of nucleic acids is the first special nucleic acid, and where the second set of nucleic acids code for either the peptide DSG or DTG, where the last nucleic acid of the second set of nucleic acids is the last special nucleic acid, with the proviso that the nucleic acids disclosed in SEQ ID NO. 1 and SEQ ID NO. 3 are not included. In a preferred embodiment, the two sets of special nucleic acids are separated by nucleic acids that code for about 125 to 222 amino acid positions, which may be any amino acids. In a highly preferred embodiment, the two sets of special nucleic acids are separated by nucleic acids that code for about 150 to 196, or 150-190, or 150 to 172 amino acid positions, which may be any amino acids. In a particular preferred embodiment, the two sets are separated by nucleic acids that code for about 172 amino acid positions, which may be any amino acids. An exemplary nucleic acid polynucleotide comprises the acid nucleotide sequence in SEQ ID NO. 5. In another particular preferred embodiment, the two sets are separated by nucleic acids that code for about 196 amino acids. An exemplary polynucleotide comprises the nucleotide sequence in SEQ ID NO. 5. In another particular embodiment, the two sets of nucleotides are separated by nucleic acids that code for about 190 amino acids. An exemplary polynucleotide

comprises the nucleotide sequence in SEQ ID NO. 1. Preferably, the first nucleic acid of the first special set of amino acids, that is, the first special nucleic acid, is operably linked to any codon where the nucleic acids of that codon codes for any peptide comprising from 1 to 10,000 amino acid (positions). In one variation, the first special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: any reporter proteins or proteins which facilitate purification. For example, the first special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathione S transferase, Green Fluorescent protein, and ubiquitin. In another variation, the last nucleic acid of the second set of special amino acids, that is, the last special nucleic acid, is operably linked to nucleic acid polymers that code for any peptide comprising any amino acids from 1 to 10,000 amino acids. In still another variation, the last special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: any reporter proteins or proteins which facilitate purification. For example, the last special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathione S transferase, Green Fluorescent protein, and ubiquitin.

20 In a related aspect, the invention provides any isolated or purified nucleic acid polynucleotide that codes for a protease capable of cleaving the beta secretase cleavage site of APP that contains two or more sets of special nucleic acids, where the special nucleic acids are separated by nucleic acids that code for about 100 to 300 amino acid positions, where the amino acids in those positions may be any amino  
25 acids, where the first set of special nucleic acids consists of the nucleic acids that code for DTG, where the first nucleic acid of the first special set of nucleic acids is the first special nucleic acid, and where the second set of nucleic acids code for either DSG or DTG, where the last nucleic acid of the second set of special nucleic acids is the last special nucleic acid, where the first special nucleic acid is operably linked to nucleic  
30 acids that code for any number of amino acids from zero to 81 amino acids and where

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codons. In a highly preferred embodiment, the polynucleotide comprises a sequence that is at least 95% identical to aspartyl-protease encoding sequences taught herein. In one variation, the second set of special nucleic acids code for the peptide DSG. In another variation, the first set of nucleic acid polynucleotide is operably linked to a peptide purification tag. For example, the nucleic acid polynucleotide is operably linked to a peptide purification tag which is six histidine. In still another variation, the first set of special nucleic acids are on one polynucleotide and the second set of special nucleic acids are on a second polynucleotide, where both first and second polynucleotides have at least 50 codons. In one embodiment of this type, both of the polynucleotides are in the same solution. In a related aspect, the invention provides a vector which contains a polynucleotide as described above, or a cell or cell line which is transformed or transfected with a polynucleotide as described above or with a vector containing such a polynucleotide.

In still another aspect, the invention provides an isolated or purified peptide or protein comprising an amino acid polymer that is a protease capable of cleaving the beta ( $\beta$ ) secretase cleavage site of APP that contains two or more sets of special amino acids, where the special amino acids are separated by about 100 to 300 amino acid positions, where each amino acid position can be any amino acid, where the first set of special amino acids consists of the peptide DTG, where the first amino acid of the first special set of amino acids is, the first special amino acid, where the second set of amino acids is selected from the peptide comprising either DSG or DTG, where the last amino acid of the second set of special amino acids is the last special amino acid, with the proviso that the proteases disclosed in SEQ ID NO. 2 and SEQ ID NO. 4 are not included. In preferred embodiments, the two sets of amino acids are separated by about 125 to 222 amino acid positions or about 150 to 196 amino acids, or about 150-190 amino acids, or about 150 to 172 amino acids, where in each position it may be any amino acid. In a particular embodiment, the two sets of amino acids are separated by about 172 amino acids. For example, the protease has the amino acid sequence described in SEQ ID NO 6. In another particular embodiment, the two sets of amino acids are separated by about 196 amino acids. For example, the two sets of amino

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number of amino acids from zero to 81 amino acid positions where in each position it may be any amino acid. In a preferred embodiment, the first special amino acid is operably linked to a peptide from about 30-77 or about 64 to 77 amino acids positions where each amino acid position may be any amino acid. In a particular embodiment, the first special amino acid is operably linked to a peptide 35, 47, 71, or 77 amino acids. In a very particular embodiment, the first special amino acid is operably linked to 71 amino acids and the first of those 71 amino acids is the amino acid T. For example, the polypeptide comprises a sequence that is at least 95% identical to an aspartyl protease sequence as described herein. In another embodiment, the first special amino acid is operably linked to any number of from 40 to 54 amino acids (positions) where each amino acid position may be any amino acid. In a particular embodiment, the first special amino acid is operably linked to amino acids that code for a peptide of 47 amino acids. In a very particular embodiment, the first special amino acid is operably linked to a 47 amino acid peptide where the first those 47 amino acids is the amino acid E. In another particular embodiment, the first special amino acid is operably linked to the same corresponding peptides from SEQ ID NO. 3 that are 35, 47, 71, or 77 peptides in length, beginning counting with the amino acids on the first special sequence, DTG, towards the N-terminal of SEQ ID NO. 3. In another particular embodiment, the polypeptide comprises a sequence that is at least 95% identical to the same corresponding amino acids in SEQ ID NO. 4, that is, identical to that portion of the sequences in SEQ ID NO. 4, including all the sequences from both the first and or the second special nucleic acids, toward the – terminal, through and including 71, 47, 35 amino acids before the first special amino acids. For example, the complete polypeptide comprises the peptide of 71 amino acids, where the first of the amino acid is T and the second is Q.

In still another related aspect, the invention provides any isolated or purified amino acid polypeptide that is a protease capable of cleaving the beta ( $\beta$ ) secretase cleavage site of APP that contains two or more sets of special amino acids, where the special amino acids are separated by about 100 to 300 amino acid positions, where each amino acid in each position can be any amino acid, where the first set of special



amino acids consists of the amino acids that code for DTG, where the first amino acid of the first special set of amino acids is, the first special amino acid, D, and where the second set of amino acids are either DSG or DTG, where the last amino acid of the second set of special amino acids is the last special amino acid, G, which is operably  
5 linked to any number of amino acids from 50 to 170 amino acids, which may be any amino acids. In preferred embodiments, the last special amino acid is operably linked to a peptide of about 100 to 170 amino acids or about 142-163 amino acids. In particular embodiments, the last special amino acid is operably linked to a peptide of about 142 amino acids, or about 163 amino acids, or about 170 amino acids. For  
10 example, the polypeptide comprises a sequence that is at least 95% identical (and preferably 100% identical) to an aspartyl protease sequence as described herein. In one particular embodiment, the second set of special amino acids is comprised of the peptide with the amino acid sequence DSG. Optionally, the amino acid polypeptide is operably linked to a peptide purification tag, such as purification tag which is six  
15 histidine. In one variation, the first set of special amino acids are on one polypeptide and the second set of special amino acids are on a second polypeptide, where both first and second polypeptide have at least 50 amino acids, which may be any amino acids. In one embodiment of this type, both of the polypeptides are in the same vessel. The invention further includes a process of making any of the polynucleotides,  
20 vectors, or cells described herein; and a process of making any of the polypeptides described herein.

In yet another related aspect, the invention provides a purified polynucleotide comprising a nucleotide sequence that encodes a polypeptide having aspartyl protease activity, wherein the polypeptide has an amino acid sequence characterized by: (a) a  
25 first tripeptide sequence DTG; (b) a second tripeptide sequence selected from the group consisting of DSG and DTG; and (c) about 100 to 300 amino acids separating the first and second tripeptide sequences, wherein the polypeptide cleaves the beta secretase cleavage site of amyloid protein precursor. In one embodiment, the polypeptide comprises an amino acid sequence depicted in SEQ ID NO: 2 or 4,  
30 whereas in another embodiment, the polypeptide comprises an amino acid sequence

other than the amino acid sequences set forth in SEQ ID NOs: 2 and 4. Similarly, the invention provides a purified polynucleotide comprising a nucleotide sequence that encodes a polypeptide that cleaves the beta secretase cleavage site of amyloid protein precursor; wherein the polynucleotide includes a strand that hybridizes to one or more  
5 of SEQ ID NOs: 3, 5, and 7 under the following hybridization conditions: hybridization overnight at 42°C for 2.5 hours in 6 X SSC/0.1% SDS, followed by washing in 1.0 X SSC at 65°C, 0.1% SDS. In one embodiment, the polypeptide comprises an amino acid sequence depicted in SEQ ID NO: 2 or 4, whereas in another embodiment, the polypeptide comprises an amino acid sequence other than the amino  
10 acid sequences set forth in SEQ ID NOs: 2 and 4. Likewise, the invention provides a purified polypeptide having aspartyl protease activity, wherein the polypeptide is encoded by polynucleotides as described in the preceding sentences. The invention also provides a vector or host cell comprising such polynucleotides, and a method of making the polypeptides using the vectors or host cells to recombinantly express the  
15 polypeptide.

In yet another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide, said polynucleotide encoding a Hu-Asp polypeptide and having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

- 20 (a) a nucleotide sequence encoding a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), wherein said Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) polypeptides have the complete amino acid sequence of SEQ ID NO. 2, SEQ ID NO. 4, and SEQ ID NO. 6, respectively; and  
(b) a nucleotide sequence complementary to the nucleotide  
25 sequence of (a).

Several species are particularly contemplated. For example, the invention provides a nucleic acid and molecule wherein said Hu-Asp polypeptide is Hu-Asp1, and said polynucleotide molecule of 1(a) comprises the nucleotide sequence of SEQ ID NO. 1; and a nucleic acid molecule wherein said Hu-Asp polypeptide is  
30 Hu-Asp2(a), and said polynucleotide molecule of 1(a) comprises the nucleotide

sequence of SEQ ID NO. 4; and a nucleic acid molecule wherein said Hu-Asp polypeptide is Hu-Asp2(b), and said polynucleotide molecule of 1(a) comprises the nucleotide sequence of SEQ ID NO. 5. In addition to the foregoing, the invention provides an isolated nucleic acid molecule comprising polynucleotide which hybridizes under stringent conditions to a polynucleotide having the nucleotide sequence in (a) or (b) as described above.

Additionally, the invention provides a vector comprising a nucleic acid molecule as described in the preceding paragraph. In a preferred embodiment, the nucleic acid molecule is operably linked to a promoter for the expression of a Hu-Asp polypeptide. Individual vectors which encode Hu-Asp1, and Hu-Asp2(a), and Hu-Asp2(b) are all contemplated. Likewise, the invention contemplates a host cell comprising any of the foregoing vectors, as well as a method of obtaining a Hu-Asp polypeptide comprising culturing such a host cell and isolating the Hu-Asp polypeptide. Host cells of the invention include bacterial cells, such as *E. coli*, and eukaryotic cells. Among the eukaryotic cells that are contemplated are insect cells, such as sf9 or High 5 cells; and mammalian cells, such as human, rodent, lagomorph, and primate. Preferred human cells include HEK293, and IMR-32 cells. Other preferred mammalian cells include COS-7, CHO-K1, Neuro-2A, and 3T3 cells. Also among the eukaryotic cells that are contemplated are a yeast cell and an avian cell.

In a related aspect, the invention provides an isolated Hu-Asp1 polypeptide comprising an amino acid sequence at least 95% identical to a sequence comprising the amino acid sequence of SEQ ID NO. 2. The invention also provides an isolated Hu-Asp2(a) polypeptide comprising an amino acid sequence at least 95% identical to a sequence comprising the amino acid sequence of SEQ ID NO. 4. The invention also provides an isolated Hu-Asp2(a) polypeptide comprising an amino acid sequence at least 95% identical to a sequence comprising the amino acid sequence of SEQ ID NO. 8.

In still another aspect, the invention provides an isolated antibody that binds specifically to any Hu-Asp polypeptide described herein, especially the polypeptide described in the preceding paragraphs.



comprises the amino acid sequence set forth in SEQ ID NO. 16. The invention further includes APP isoform variants as set forth in SEQ ID NOs. 18 and 20. The invention further includes all polynucleotides that encode an APP protein that has been modified to include two C-terminal lysines; as well as any eukaryotic cell line comprising such nucleic acids or polypeptides. Preferred cell lines include a mammalian cell line (*e.g.*, HEK293, Neuro2a).

Thus, in one embodiment, the invention provides a polypeptide comprising the amino acid sequence of a mammalian amyloid protein precursor (APP) or fragment thereof containing an APP cleavage site recognizable by a mammalian  $\beta$ -secretase, and further comprising two lysine residues at the carboxyl terminus of the amino acid sequence of the mammalian APP or APP fragment. As taught herein in detail, the addition of two additional lysine residues to APP sequences has been found to greatly increase A $\beta$  processing of the APP in APP processing assays. Thus, the di-lysine modified APP reagents of the invention are particularly useful in assays to identify modulators of A $\beta$  production, for use in designing therapeutics for the treatment or prevention of Alzheimer's disease. In one embodiment, the polypeptide comprises the complete amino acid sequence of a mammalian amyloid protein precursor (APP), and further comprises the two lysine residues at the carboxyl terminus of the amino acid sequence of the mammalian amyloid protein precursor. In an alternative embodiment, the polypeptide comprises only a fragment of the APP, the fragment containing at least that portion of APP that is cleaved by a mammalian  $\beta$ -secretase in the formation of A $\beta$  peptides.

The practice of assays that monitor cleavage of APP can be facilitated by attaching a marker to a portion of the APP. Measurement of retained or liberated marker can be used to quantitate the amount of APP cleavage that occurs in the assay, *e.g.*, in the presence or absence of a putative modulator of cleavage activity. Thus, in one preferred embodiment, the polypeptide of the invention further includes a marker. For example, the marker comprises a reporter-protein amino acid sequence attached to the APP amino acid sequence. Exemplary reporter proteins include a fluorescing protein (*e.g.*, green fluorescing proteins, luciferase) or an enzyme that is used to

cleave a substrate to produce a colorimetric cleavage product. Also contemplated are tag sequences which are commonly used as epitopes for quantitative immunoassays.

In a preferred embodiment, the di-lysine-modified APP of the invention is a human APP. For example, human APP isoforms such as APP695, APP751, and APP770, modified to include the two lysines, are contemplated. In a preferred embodiment, the APP isoform comprises at least one variation selected from the group consisting of a Swedish KM-NL mutation and a London V717-F mutation, or any other mutation that has been observed in a subpopulation that is particularly prone to development of Alzheimer's disease. These mutations are recognized as mutations that influence APP processing into A $\beta$ . In a highly preferred embodiment, the APP protein or fragment thereof comprises the APP-Sw  $\beta$ -secretase peptide sequence NLDA (SEQ ID NO: 66), which is associated with increased levels of A $\beta$  processing and therefore is particularly useful in assays relating to Alzheimer's research. More particularly, the APP protein or fragment thereof preferably comprises the APP-Sw  $\beta$ -secretase peptide sequence SEVNLDAEFR (SEQ ID NO: 63).

In one preferred embodiment, the APP protein or fragment thereof further includes an APP transmembrane domain carboxy-terminal to the APP-Sw  $\beta$ -secretase peptide sequence. Polypeptides that include the TM domain are particularly useful in cell-based APP processing assays. In contrast, embodiments lacking the TM domain are useful in cell-free assays of APP processing.

In addition to working with APP from humans and various animal models, researchers in the field of Alzheimer's also have construct chimeric APP polypeptides which include stretches of amino acids from APP of one species (e.g., humans) fused to stretches of APP from one or more other species (e.g., rodent). Thus, in another embodiment of the polypeptide of the invention, the APP protein or fragment thereof comprises a chimeric APP, the chimeric APP including partial APP amino acid sequences from at least two species. A chimeric APP that includes amino acid sequence of a human APP and a rodent APP is particularly contemplated.

In a related aspect, the invention provides a polynucleotide comprising a nucleotide sequence that encodes a polypeptide as described in the preceding

paragraphs. Such a polynucleotide is useful for recombinant expression of the polypeptide of the invention for use in APP processing assays. In addition, the polynucleotide is useful for transforming into cells to produce recombinant cells that express the polypeptide of the invention, which cells are useful in cell-based assays to identify modulators of APP processing. Thus, in addition to polynucleotides, the invention provides a vector comprising such polynucleotides, especially expression vectors where the polynucleotide is operably linked to a promoter to promote expression of the polypeptide encoded by the polynucleotide in a host cell. The invention further provides a host cell transformed or transfected with a polynucleotide according to claim 14 or a vector according to claim 15 or 16. Among the preferred host cells are mammalian cells, especially human cells.

In another, related embodiment, the invention provides a polypeptide useful for assaying for modulators of  $\beta$ -secretase activity, said polypeptide comprising an amino acid sequence of the formula  $\text{NH}_2\text{-X-Y-Z-KK-COOH}$ ; wherein X, Y, and Z each comprise an amino acid sequence of at least one amino acid; wherein  $\text{-NH}_2\text{-X}$  comprises an amino-terminal amino acid sequence having at least one amino acid residue; wherein Y comprises an amino acid sequence of a  $\beta$ -secretase recognition site of a mammalian amyloid protein precursor (APP); and wherein  $\text{Z-KK-COOH}$  comprises a carboxy-terminal amino acid sequence ending in two lysine (K) residues.

In one preferred variation, the carboxyl-terminal amino acid sequence Z includes a hydrophobic domain that is a transmembrane domain in host cells that express the polypeptide. Host cells that express such a polypeptide are particularly useful in assays described herein for identifying modulators of APP processing. In another preferred variation, the amino-terminal amino acid sequence X includes an amino acid sequence of a reporter or marker protein, as described above. In still another preferred variation, the  $\beta$ -secretase recognition site Y comprises the human APP-Sw  $\beta$ -secretase peptide sequence NLDA (SEQ ID NO: 66). It will be apparent that these preferred variations are not mutually exclusive of each other -- they may be combined in a single polypeptide. The invention further provides a polynucleotide comprising a nucleotide sequence that encodes such polypeptides, vectors which comprise such

polynucleotides, and host cells which comprises such vectors, polynucleotides, and/or polypeptides.

In yet another aspect, the invention provides a method for identifying inhibitors of an enzyme that cleaves the beta secretase cleavable site of APP comprising:

- a) culturing cells in a culture medium under conditions in which the enzyme causes processing of APP and release of amyloid beta-peptide into the medium and causes the accumulation of CTF99 fragments of APP in cell lysates,
- b) exposing the cultured cells to a test compound; and specifically determining whether the test compound inhibits the function of the enzyme by measuring the amount of amyloid beta-peptide released into the medium and/or the amount of CTF99 fragments of APP in cell lysates;
- c) identifying test compounds diminishing the amount of soluble amyloid beta peptide present in the culture medium and diminution of CTF99 fragments of APP in cell lysates as Asp2 inhibitors. In preferred embodiments, the cultured cells are a human, rodent or insect cell line. It is also preferred that the human or rodent cell line exhibits  $\beta$  secretase activity in which processing of APP occurs with release of amyloid beta-peptide into the culture medium and accumulation of CTF99 in cell lysates. Among the contemplated test compounds are antisense oligomers directed against the enzyme that exhibits  $\beta$  secretase activity, which oligomers reduce release of soluble amyloid beta-peptide into the culture medium and accumulation of CTF99 in cell lysates.

In yet another aspect, the invention provides a method for the identification of an agent that decreases the activity of a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method comprising:

- a) determining the activity of said Hu-Asp polypeptide in the presence of a test agent and in the absence of a test agent; and
- b) comparing the activity of said Hu-Asp polypeptide determined in the presence of said test agent to the activity of said Hu-Asp polypeptide determined in the absence of said test agent; whereby a lower level of activity in the presence of said



test agent than in the absence of said test agent indicates that said test agent has decreased the activity of said Hu-Asp polypeptide.

In a related aspect, the invention provides a method for assaying for modulators of  $\beta$ -secretase activity, comprising the steps of:

- 5 (a) contacting a first composition with a second composition both in the presence and in the absence of a putative modulator compound, wherein the first composition comprises a mammalian  $\beta$ -secretase polypeptide or biologically active fragment thereof, and wherein the second composition comprises a substrate polypeptide having an amino acid sequence comprising a  $\beta$ -secretase cleavage site;
- 10 (b) measuring cleavage of the substrate polypeptide in the presence and in the absence of the putative modulator compound; and (c) identifying modulators of  $\beta$ -secretase activity from a difference in cleavage in the presence versus in the absence of the putative modulator compound. A modulator that is a  $\beta$ -secretase antagonist (inhibitor) reduces such cleavage, whereas a modulator that is a  $\beta$ -secretase agonist
- 15 increases such cleavage. Since such assays are relevant to development of Alzheimer's disease therapeutics for humans, it will be readily apparent that, in one preferred embodiment, the first composition comprises a purified human Asp2 polypeptide. In one variation, the first composition comprises a soluble fragment of a human Asp2 polypeptide that retains Asp2  $\beta$ -secretase activity. Several such
- 20 fragments (including  $\Delta$ TM fragments) are described herein in detail. Thus, in a particular embodiment, the soluble fragment is a fragment lacking an Asp2 transmembrane domain.

The  $\beta$ -secretase cleavage site in APP is known, and it will be appreciated that the oassays of the invention can be performed with either intact APP

25 or fragments or analogs of APP that retain the  $\beta$ -secretase recognition and cleavage site. Thus, in one variation, the substrate polypeptide of the second composition comprises the amino acid sequence SEVNLDAEFR (SEQ ID NO: 63), which includes the  $\beta$ -secretase recognition site of human APP that contains the "Swiss" mutation. In another variation, the substrate polypeptide of the second composition

30 comprises the amino acid sequence EVKMDAEF (SEQ ID NO: 67). In another

variation, the second composition comprises a polypeptide having an amino acid sequence of a human amyloid precursor protein (APP). For example, the human amyloid precursor protein is selected from the group consisting of: APP695, APP751, and APP770. Preferably, the human amyloid precursor protein (irrespective of  
5 isoform selected) includes at least one mutation selected from a KM-NL Swiss mutation and a V-F London mutation. As explained elsewhere, one preferred embodiment involves a variation wherein the polypeptide having an amino acid sequence of a human APP further comprises an amino acid sequence comprising a marker sequence attached amino-terminal to the amino acid sequence of the human  
10 amyloid precursor protein. Preferably, the polypeptide having an amino acid sequence of a human APP further comprises two lysine residues attached to the carboxyl terminus of the amino acid sequence of the human APP. The assays can be performed in a cell free setting, using cell-free enzyme and cell-free substrate, or can be performed in a cell-based assay wherein the second composition comprises a  
15 eukaryotic cell that expresses amyloid precursor protein (APP) or a fragment thereof containing a  $\beta$ -secretase cleavage site. Preferably, the APP expressed by the host cell is an APP variant that includes two carboxyl-terminal lysine residues. It will also be appreciated that the  $\beta$ -secretase enzyme can be an enzyme that is expressed on the surface of the same cells.

20 The present invention provides isolated nucleic acid molecules comprising a polynucleotide that codes for a polypeptide selected from the group consisting of human aspartyl proteases. In particular, human aspartyl protease 1 (Hu-Asp1) and two alternative splice variants of human aspartyl protease-2 (Hu-Asp2), a "long" (L) form designated herein as Hu-Asp2(a) and a "short" (S) form designated Hu-Asp2(b).  
25 As used herein, all references to "Hu-Asp" should be understood to refer to all of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b). In addition, as used herein, all references to "Hu-Asp2" should be understood to refer to both Hu-Asp2(a) and Hu-Asp2(b). Hu-Asp1 is expressed most abundantly in pancreas and prostate tissues, while Hu-Asp2(a) and Hu-Asp2(b) are expressed most abundantly in pancreas and brain

tissues. The invention also provides isolated Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as fragments thereof which exhibit aspartyl protease activity.

In a preferred embodiment, the nucleic acid molecules comprise a polynucleotide having a nucleotide sequence selected from the group consisting of residues 1-1554 of SEQ ID NO. 1, encoding Hu-Asp1, residues 1-1503 of SEQ ID NO. 3, encoding Hu-Asp2(a), and residues 1-1428 of SEQ ID NO.5, encoding Hu-Asp2(b). In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide encoding Hu-Asp1, Hu-Asp2(a), Hu-Asp-2(b), or fragments thereof. European patent application EP 0 848 062 discloses a polypeptide referred to as "Asp 1," that bears substantial homology to Hu-Asp1, while international application WO 98/22597 discloses a polypeptide referred to as "Asp 2," that bears substantial homology to Hu-Asp2(a).

The present invention also provides vectors comprising the isolated nucleic acid molecules of the invention, host cells into which such vectors have been introduced, and recombinant methods of obtaining a Hu-Asp1, Hu-Asp2(a), or Hu-Asp2(b) polypeptide comprising culturing the above-described host cell and isolating the relevant polypeptide.

In another aspect, the invention provides isolated Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as fragments thereof. In a preferred embodiment, the Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides have the amino acid sequence given in SEQ ID NO. 2, SEQ ID NO. 4, or SEQ ID NO.6, respectively. The present invention also describes active forms of Hu-Asp2, methods for preparing such active forms, methods for preparing soluble forms, methods for measuring Hu-Asp2 activity, and substrates for Hu-Asp2 cleavage. The invention also describes antisense oligomers targeting the Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) mRNA transcripts and the use of such antisense reagents to decrease such mRNA and consequently the production of the corresponding polypeptide. Isolated antibodies, both polyclonal and monoclonal, that binds specifically to any of the Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides of the invention are also provided.

The invention also provides a method for the identification of an agent that modulates the activity of any of Hu-Asp-1, Hu-Asp2(a), and Hu-Asp2(b). The inventions

describes methods to test such agents in cell-free assays to which Hu-Asp2 polypeptide is added, as well as methods to test such agents in human or other mammalian cells in which Hu-Asp2 is present.

5 Additional features and variations of the invention will be apparent to those skilled in the art from the entirety of this application, including the drawing and detailed description, and all such features are intended as aspects of the invention. Likewise, features of the invention described herein can be re-combined into additional embodiments that are also intended as aspects of the invention, irrespective of whether the combination of features is specifically mentioned above as an aspect or embodiment of the invention. Also, only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the invention.

10 In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations specifically mentioned above. Although the applicant(s) invented the full scope of the claims appended hereto, the claims appended hereto are not intended to encompass within their scope the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention.

## 25 **BRIEF DESCRIPTION OF THE SEQUENCE LISTING**

Sequence ID No. 1: Human Asp-1, nucleotide sequence.

Sequence ID No. 2: Human Asp-1, predicted amino acid sequence.

Sequence ID No. 3: Human Asp-2(a), nucleotide sequence.

30 Sequence ID No. 4: Human Asp-2(a), predicted amino acid sequence. The Asp2(a) amino acid sequence includes a putative signal peptide comprising residues 1

to 21; and a putative pre-propeptide after the signal peptide that extends through residue 45 (as assessed by processing observed of recombinant Asp2(a) in CHO cells), and a putative propeptide that may extend to at least about residue 57, based on the observation of an observed GRRIGS (SEQ ID NO: 68) sequence which has characteristics of a protease recognition sequence. The Asp2(a) further includes a transmembrane domain comprising residues 455-477, a cytoplasmic domain comprising residues 478-501, and a putative alpha-helical spacer region, comprising residues 420-454, believed to be unnecessary for proteolytic activity, between the protease catalytic domain and the transmembrane domain.

Sequence ID No. 5: Human Asp-2(b), nucleotide sequence.

Sequence ID No. 6: Human Asp-2(b), predicted amino acid sequence. The Asp2(b) amino acid sequence includes a putative signal peptide, pre-propeptide, and propeptide as described above for Asp2(a). The Asp2(b) further includes a transmembrane domain comprising residues 430-452, a cytoplasmic domain comprising residues 453-476, and a putative alpha-helical spacer region, comprising residues 395-429, believed to be unnecessary for proteolytic activity, between the protease catalytic domain and the transmembrane domain.

Sequence ID No. 7: Murine Asp-2(a), nucleotide sequence.

Sequence ID No. 8: Murine Asp-2(a), predicted amino acid sequence. The proteolytic processing of murine Asp2(a) is believed to be analogous to the processing described above for human Asp2(a). In addition, a variant lacking amino acid residues 190-214 of SEQ ID NO: 8 is specifically contemplated as a murine Asp2(b) polypeptide.

Sequence ID No. 9: Human APP695, nucleotide sequence.

Sequence ID No.10: Human APP695, predicted amino acid sequence.

Sequence ID No.11: Human APP695-Sw, nucleotide sequence.

Sequence ID No.12: Human APP695-Sw. predicted amino acid sequence. In the APP695 isoform, the Sw mutation is characterized by a KM-NL alteration at positions 595-596 (compared to normal APP695).

Sequence ID No.13: Human APP695-VF, nucleotide sequence.

Sequence ID No.14: Human APP695-VF, predicted amino acid sequence. In the APP 695 isoform, the VF mutation is characterized by a V-F alteration at position 642 (compared to normal APP 695).

Sequence ID No.15: Human APP695-KK, nucleotide sequence.

5      Sequence ID No.16: Human APP695-KK, predicted amino acid sequence.  
(APP695 with two carboxy-terminal lysine residues.)

Sequence ID No.17: Human APP695-Sw-KK, nucleotide sequence.

Sequence ID No.18: Human APP695-Sw-KK, predicted amino acid sequence

Sequence ID No.19: Human APP695-VF-KK, nucleotide sequence

10      Sequence ID No.20: Human APP695-VF-KK, predicted amino acid sequence

Sequence ID No.21: T7-Human-pro-Asp-2(a) $\Delta$ TM, nucleotide sequence

Sequence ID No.22: T7-Human-pro-Asp-2(a) $\Delta$ TM, amino acid sequence

Sequence ID No.23: T7-Caspase-Human-pro-Asp-2(a) $\Delta$ TM, nucleotide  
sequence

15      Sequence ID No.24: T7-Caspase-Human-pro-Asp-2(a) $\Delta$ TM, amino acid  
sequence

Sequence ID No.25: Human-pro-Asp-2(a) $\Delta$ TM (low GC), nucleotide  
sequence

20      Sequence ID No.26: Human-pro-Asp-2(a) $\Delta$ TM, (low GC), amino acid  
sequence

Sequence ID No.27: T7-Caspase-Caspase 8  
cleavage-Human-pro-Asp-2(a) $\Delta$ TM, nucleotide sequence

Sequence ID No.28: T7-Caspase-Caspase 8  
cleavage-Human-pro-Asp-2(a) $\Delta$ TM, amino acid sequence

25      Sequence ID No.29: Human Asp-2(a) $\Delta$ TM, nucleotide sequence

Sequence ID No.30: Human Asp-2(a) $\Delta$ TM, amino acid sequence

Sequence ID No.31: Human Asp-2(a) $\Delta$ TM(His)<sub>6</sub>, nucleotide sequence

Sequence ID No. 32: Human Asp-2(a) $\Delta$ TM(His)<sub>6</sub>, amino acid sequence

30      Sequence ID Nos. 33-49 are short synthetic peptide and oligonucleotide  
sequences that are described below in the Detailed Description of the Invention.

Sequence ID No. 50: Human Asp2(b) $\Delta$ TM polynucleotide sequence.

Sequence ID No. 51: Human Asp2(b) $\Delta$ TM polypeptide sequence (exemplary variant of Human Asp2(b) lacking transmembrane and intracellular domains of Hu-Asp2(b) set forth in SEQ ID NO: 6.

5                    Sequence ID No. 52: Human Asp2(b) $\Delta$ TM(His)<sub>6</sub> polynucleotide sequence.

Sequence ID No. 53: Human Asp2(b)ΔTM(His)<sub>6</sub> polypeptide sequence  
(Human Asp2(b)ΔTM with six histidine tag attached to C-terminus)

**Sequence ID No. 54: Human APP770-encoding polynucleotide sequence.**

Sequence ID No. 55: Human APP770 polypeptide sequence. To introduce the  
10 KM-NL Swedish mutation, residues KM at positions 670-71 are changed to NL. To  
introduce the V-F London mutation, the V residue at position 717 is changed to F.

**Sequence ID No. 56: Human APP751 encoding polynucleotide sequence.**

Sequence ID No. 57: Human APP751 polypeptide sequence (Human APP751 isoform).

15 Sequence ID No. 58: Human APP770-KK encoding polynucleotide sequence.

Sequence ID No. 59: Human APP770-KK polypeptide sequence. (Human APP770 isoform to which two C-terminal lysines have been added).

Sequence ID No. 60: Human APP751-KK encoding polynucleotide sequence.

Sequence ID No. 61: Human APP751-KK polypeptide sequence (Human  
20 APP751 isoform to which two C-terminal lysines have been added).

Sequence ID No. 62-65: Various short peptide sequences described in detail in detailed description.

### BRIEF DESCRIPTION OF THE FIGURES

25                      Figure 1:              Figure 1 shows the nucleotide (SEQ ID NO:1) and predicted amino acid sequence (SEQ ID NO:2) of human Asp1.

Figure 2: Figure 2 shows the nucleotide (SEQ ID NO:3) and predicted amino acid sequence (SEQ ID NO:4) of human Asp2(a).

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Alanine, Ala, A; Arginine, Arg, R; Asparagine, Asn, N; Aspartic acid, Asp, D; Cysteine, Cys, C; Glutamine, Gln, Q; Glutamic Acid, Glu, E; Glycine, Gly, G; Histidine, His, H; Isoleucine, Ile, I; Leucine, Leu, L; Lysine, Lys, K; Methionine, Met, M; Phenylalanine, Phe, F; Proline, Pro, P; Serine, Ser, S; Threonine, Thr, T; 5 Tryptophan, Trp, W; Tyrosine, Tyr, Y; Valine, Val, V; Aspartic acid or Asparagine, Asx, B; Glutamic acid or Glutamine, Glx, Z; Any amino acid, Xaa, X.

The present invention describes a method to scan gene databases for the simple active site motif characteristic of aspartyl proteases. Eukaryotic aspartyl proteases such as pepsin and renin possess a two-domain structure which folds to 10 bring two aspartyl residues into proximity within the active site. These are embedded in the short tripeptide motif DTG, or more rarely, DSG. Most aspartyl proteases occur as proenzyme whose N-terminus must be cleaved for activation. The DTG or DSG active site motif appears at about residue 65-70 in the proenzyme (prorenin, pepsinogen), but at about residue 25-30 in the active enzyme after cleavage of the 15 N-terminal prodomain. The limited length of the active site motif makes it difficult to search collections of short, expressed sequence tags (EST) for novel aspartyl proteases. EST sequences typically average 250 nucleotides or less, and so would encode 80-90 amino acid residues or less. That would be too short a sequence to span the two active site motifs. The preferred method is to scan databases of hypothetical 20 or assembled protein coding sequences. The present invention describes a computer method to identify candidate aspartyl proteases in protein sequence databases. The method was used to identify seven candidate aspartyl protease sequences in the *Caenorhabditis elegans* genome. These sequences were then used to identify by 25 homology search Hu-Asp1 and two alternative splice variants of Hu-Asp2, designated herein as Hu-Asp2(a) and Hu-Asp2(b).

In a major aspect of the invention disclosed here we provide new information about APP processing. Pathogenic processing of the amyloid precursor protein (APP) via the A $\beta$  pathway requires the sequential action of two proteases referred to as  $\beta$ -secretase and  $\gamma$ -secretase. Cleavage of APP by the  $\beta$ -secretase and  $\gamma$ -secretase 30 generates the N-terminus and C-terminus of the A $\beta$  peptide, respectively. Because



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The predicted amino acid sequences of Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) share significant homology with previously identified mammalian aspartyl proteases such as pepsinogen A, pepsinogen B, cathepsin D, cathepsin E, and renin. P.B.Szeacs, *Scand. J. Clin. Lab. Invest.* 52:(Suppl. 210 5-22 (1992)). These enzymes are  
5 characterized by the presence of a duplicated DTG/DSG sequence motif. The Hu-Asp1 and HuAsp2 polypeptides disclosed herein also exhibit extremely high homology with the ProSite consensus motif for aspartyl proteases extracted from the SwissProt database.

The nucleotide sequence given as residues 1-1554 of SEQ ID NO:1  
10 corresponds to the nucleotide sequence encoding Hu-Asp1, the nucleotide sequence given as residues 1-1503 of SEQ ID NO:3 corresponds to the nucleotide sequence encoding Hu-Asp2(a), and the nucleotide sequence given as residues 1-1428 of SEQ ID NO:5 corresponds to the nucleotide sequence encoding Hu-Asp2(b). The isolation and sequencing of DNA encoding Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) is  
15 described below in Examples 1 and 2.

As is described in Examples 1 and 2, automated sequencing methods were used to obtain the nucleotide sequence of Hu-Asp1, Hu-Asp2(a), and Hu-Asp-2(b). The Hu-Asp nucleotide sequences of the present invention were obtained for both DNA strands, and are believed to be 100% accurate. However, as is known in the art,  
20 nucleotide sequence obtained by such automated methods may contain some errors. Nucleotide sequences determined by automation are typically at least about 90%, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of a given nucleic acid molecule. The actual sequence may be more precisely determined using manual sequencing methods, which are well known  
25 in the art. An error in sequence which results in an insertion or deletion of one or more nucleotides may result in a frame shift in translation such that the predicted amino acid sequence will differ from that which would be predicted from the actual nucleotide sequence of the nucleic acid molecule, starting at the point of the mutation. The Hu-Asp DNA of the present invention includes cDNA, chemically synthesized  
30 DNA, DNA isolated by PCR, genomic DNA, and combinations thereof. Genomic

Hu-Asp DNA may be obtained by screening a genomic library with the Hu-Asp2 cDNA described herein, using methods that are well known in the art, or with oligonucleotides chosen from the Hu-Asp2 sequence that will prime the polymerase chain reaction (PCR). RNA transcribed from Hu-Asp DNA is also encompassed by the present invention.

Due to the degeneracy of the genetic code, two DNA sequences may differ and yet encode identical amino acid sequences. The present invention thus provides isolated nucleic acid molecules having a polynucleotide sequence encoding any of the Hu-Asp polypeptides of the invention, wherein said polynucleotide sequence encodes a Hu-Asp polypeptide having the complete amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or fragments thereof.

Also provided herein are purified Hu-Asp polypeptides, both recombinant and non-recombinant. Most importantly, methods to produce Hu-Asp2 polypeptides in active form are provided. These include production of Hu-Asp2 polypeptides and variants thereof in bacterial cells, insect cells, and mammalian cells, also in forms that allow secretion of the Hu-Asp2 polypeptide from bacterial, insect or mammalian cells into the culture medium, also methods to produce variants of Hu-Asp2 polypeptide incorporating amino acid tags that facilitate subsequent purification. In a preferred embodiment of the invention the Hu-Asp2 polypeptide is converted to a proteolytically active form either in transformed cells or after purification and cleavage by a second protease in a cell-free system, such active forms of the Hu-Asp2 polypeptide beginning with the N-terminal sequence TQHGR (SEQ ID NO: 69) or ETDEEP (SEQ ID NO: 70). The sequence TQHGR (SEQ ID NO: 69) represents the amino-terminus of Asp2(a) or Asp2(b) beginning with residue 22 of SEQ ID NO: 4 or 6, after cleavage of a putative 21 residue signal peptide. Recombinant Asp2(a) expressed in and purified from insect cells was observed to have this amino terminus, presumably as a result of cleavage by a signal peptidase. The sequence ETDEEP (SEQ ID NO: 70) represents the amino-terminus of Asp2(a) or Asp2(b) beginning with residue 46 of SEQ ID NO: 4 or 6, as observed when Asp2(a) has been recombinantly produced in CHO cells (presumably after cleavage by both a rodent



a purified polypeptide as described in the preceding paragraph that further lacks cytoplasmic domain amino acids 478 to 501 of SEQ ID NO: 4;

5 a purified polypeptide as described in either of the preceding paragraphs that further lacks amino acids 420-454 of SEQ ID NO: 4, which constitute a putative alpha helical region between the catalytic domain and the transmembrane domain that is believed to be unnecessary for  $\beta$ -secretase activity;

10 a purified polypeptide that comprises an amino acid sequence that includes amino acids 58 to 419 of SEQ ID NO: 4, and that lacks amino acids 22 to 57 of SEQ ID NO: 4;

a purified polypeptide that comprises an amino acid sequence that includes amino acids 46 to 419 of SEQ ID NO: 4, and that lacks amino acids 22 to 45 of SEQ ID NO: 4;

15 a purified polypeptide that comprises an amino acid sequence that includes amino acids 22 to 454 of SEQ ID NO: 4.

a purified polypeptide that comprises a fragment of Asp2(b) having the amino acid sequence set forth in SEQ ID NO: 6, and wherein said polypeptide lacks transmembrane domain amino acids 430 to 452 of SEQ ID NO: 6;

20 a purified polypeptide as described in the preceding paragraph that further lacks cytoplasmic domain amino acids 453 to 476 of SEQ ID NO: 6;

25 a purified polypeptide as described in either of the preceding two paragraphs that further lacks amino acids 395-429 of SEQ ID NO: 4, which constitute a putative alpha helical region between the catalytic domain and the transmembrane domain that is believed to be unnecessary for  $\beta$ -secretase activity;

a purified polypeptide comprising an amino acid sequence that includes amino acids 58 to 394 of SEQ ID NO: 4, and that lacks amino acids 22 to 57 of SEQ ID NO: 4;



Part B7

a purified polypeptide comprising an amino acid sequence that includes amino acids 46 to 394 of SEQ ID NO: 4, and that lacks amino acids 22 to 45 of SEQ ID NO: 4; and

5 Part B8

a purified polypeptide comprising an amino acid sequence that includes amino acids 22 to 429 of SEQ ID NO: 4.

Also included as part of the invention is a purified polynucleotide comprising a nucleotide sequence that encodes such polypeptides; a vector comprising a polynucleotide that encodes such polypeptides; and a host cell transformed or transfected with such a polynucleotide or vector.

10 Hu-Asp variants may be obtained by mutation of native Hu-Asp-encoding nucleotide sequences, for example. A Hu-Asp variant, as referred to herein, is a polypeptide substantially homologous to a native Hu-Asp polypeptide but which has an amino acid sequence different from that of native Hu-Asp because of one or more deletions, insertions, or substitutions in the amino acid sequence. The variant amino  
15 acid or nucleotide sequence is preferably at least about 80% identical, more preferably at least about 90% identical, and most preferably at least about 95% identical, to a native Hu-Asp sequence. Thus, a variant nucleotide sequence which contains, for example, 5 point mutations for every one hundred nucleotides, as compared to a native Hu-Asp gene, will be 95% identical to the native protein. The  
20 percentage of sequence identity, also termed homology, between a native and a variant Hu-Asp sequence may also be determined, for example, by comparing the two sequences using any of the computer programs commonly employed for this purpose, such as the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wisconsin), which  
25 uses the algorithm of Smith and Waterman (*Adv. Appl. Math.* 2: 482-489 (1981)).

Alterations of the native amino acid sequence may be accomplished by any of a number of known techniques. For example, mutations may be introduced at particular locations by procedures well known to the skilled artisan, such as oligonucleotide-directed mutagenesis, which is described by Walder *et al.* (*Gene*  
30 42:133 (1986)); Bauer *et al.* (*Gene* 37:73 (1985)); Craik (*BioTechniques*, January

1985, pp. 12-19); Smith *et al.* (*Genetic Engineering: Principles and Methods*, Plenum Press (1981)); and U.S. Patent Nos. 4,518,584 and 4,737,462.

Hu-Asp variants within the scope of the invention may comprise conservatively substituted sequences, meaning that one or more amino acid residues of a Hu-Asp polypeptide are replaced by different residues that do not alter the secondary and/or tertiary structure of the Hu-Asp polypeptide. Such substitutions may include the replacement of an amino acid by a residue having similar physicochemical properties, such as substituting one aliphatic residue (Ile, Val, Leu or Ala) for another, or substitution between basic residues Lys and Arg, acidic residues Glu and Asp, amide residues Gln and Asn, hydroxyl residues Ser and Tyr, or aromatic residues Phe and Tyr. Further information regarding making phenotypically silent amino acid exchanges may be found in Bowie *et al.*, *Science* 247:1306-1310 (1990). Other Hu-Asp variants which might retain substantially the biological activities of Hu-Asp are those where amino acid substitutions have been made in areas outside functional regions of the protein.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent conditions to a portion of the nucleic acid molecules described above, *e.g.*, to at least about 15 nucleotides, preferably to at least about 20 nucleotides, more preferably to at least about 30 nucleotides, and still more preferably to at least about from 30 to at least about 100 nucleotides, of one of the previously described nucleic acid molecules. Such portions of nucleic acid molecules having the described lengths refer to, *e.g.*, at least about 15 contiguous nucleotides of the reference nucleic acid molecule. By stringent hybridization conditions is intended overnight incubation at about 42°C for about 2.5 hours in 6 X SSC/0.1% SDS, followed by washing of the filters four times for 15 minutes in 1.0 X SSC at 65°C, 0.1% SDS.

Fragments of the Hu-Asp encoding nucleic acid molecules described herein, as well as polynucleotides capable of hybridizing to such nucleic acid molecules may be used as a probe or as primers in a polymerase chain reaction (PCR). Such probes may be used, *e.g.*, to detect the presence of Hu-Asp nucleic acids in *in vitro* assays, as well

as in Southern and northern blots. Cell types expressing Hu-Asp may also be identified by the use of such probes. Such procedures are well known, and the skilled artisan will be able to choose a probe of a length suitable to the particular application. For PCR, 5' and 3' primers corresponding to the termini of a desired Hu-Asp nucleic acid molecule are employed to isolate and amplify that sequence using conventional techniques.

Other useful fragments of the Hu-Asp nucleic acid molecules are antisense or sense oligonucleotides comprising a single stranded nucleic acid sequence capable of binding to a target Hu-Asp mRNA (using a sense strand), or Hu-Asp DNA (using an antisense strand) sequence. In a preferred embodiment of the invention these Hu-Asp antisense oligonucleotides reduce Hu-Asp mRNA and consequent production of Hu-Asp polypeptides.

In another aspect, the invention includes Hu-Asp polypeptides with or without associated native pattern glycosylation. Both Hu-Asp1 and Hu-Asp2 have canonical acceptor sites for Asn-linked sugars, with Hu-Asp1 having two of such sites, and Hu-Asp2 having four. Hu-Asp expressed in yeast or mammalian expression systems (discussed below) may be similar to or significantly different from a native Hu-Asp polypeptide in molecular weight and glycosylation pattern. Expression of Hu-Asp in bacterial expression systems will provide non-glycosylated Hu-Asp.

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. Hu-Asp polypeptides may be recovered and purified from tissues, cultured cells, or recombinant cell cultures by well-known methods, including ammonium sulfate or ethanol precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography, and high performance liquid chromatography (HPLC). In a preferred embodiment, an amino acid tag is added to the Hu-Asp polypeptide using genetic engineering techniques that are well known to practitioners of the art which include addition of six histidine amino acid residues to allow purification by binding to nickel immobilized on a suitable support, epitopes for

polyclonal or monoclonal antibodies including but not limited to the T7 epitope, the myc epitope, and the V5a epitope, and fusion of Hu-Asp2 to suitable protein partners including but not limited to glutathione-S-transferase or maltose binding protein. In a preferred embodiment these additional amino acid sequences are added to the C-terminus of Hu-Asp but may be added to the N-terminus or at intervening positions within the Hu-Asp2 polypeptide.

The present invention also relates to vectors comprising the polynucleotide molecules of the invention, as well as host cell transformed with such vectors. Any of the polynucleotide molecules of the invention may be joined to a vector, which generally includes a selectable marker and an origin of replication, for propagation in a host. Because the invention also provides Hu-Asp polypeptides expressed from the polynucleotide molecules described above, vectors for the expression of Hu-Asp are preferred. The vectors include DNA encoding any of the Hu-Asp polypeptides described above or below, operably linked to suitable transcriptional or translational regulatory sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the DNA encoding Hu-Asp. Thus, a promoter nucleotide sequence is operably linked to a Hu-Asp DNA sequence if the promoter nucleotide sequence directs the transcription of the Hu-Asp sequence.

Selection of suitable vectors to be used for the cloning of polynucleotide molecules encoding Hu-Asp, or for the expression of Hu-Asp polypeptides, will of course depend upon the host cell in which the vector will be transformed, and, where applicable, the host cell from which the Hu-Asp polypeptide is to be expressed. Suitable host cells for expression of Hu-Asp polypeptides include prokaryotes, yeast, and higher eukaryotic cells, each of which is discussed below.

30 The Hu-Asp polypeptides to be expressed in such host cells may also be fusion proteins which include regions from heterologous proteins. Such regions may be included to allow, *e.g.*, secretion, improved stability, or facilitated purification of the

polypeptide. For example, a sequence encoding an appropriate signal peptide can be incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused inframe to the Hu-Asp sequence so that Hu-Asp is translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cell promotes extracellular secretion of the Hu-Asp polypeptide.

Preferably, the signal sequence will be cleaved from the Hu-Asp polypeptide upon secretion of Hu-Asp from the cell. Nonlimiting examples of signal sequences that can be used in practicing the invention include the yeast Ifactor and the honeybee melatin leader in sf9 insect cells.

In a preferred embodiment, the Hu-Asp polypeptide will be a fusion protein which includes a heterologous region used to facilitate purification of the polypeptide. Many of the available peptides used for such a function allow selective binding of the fusion protein to a binding partner. For example, the Hu-Asp polypeptide may be modified to comprise a peptide to form a fusion protein which specifically binds to a binding partner, or peptide tag. Nonlimiting examples of such peptide tags include the 6-His tag, thioredoxin tag, hemagglutinin tag, GST tag, and OmpA signal sequence tag. As will be understood by one of skill in the art, the binding partner which recognizes and binds to the peptide may be any molecule or compound including metal ions (*e.g.*, metal affinity columns), antibodies, or fragments thereof, and any protein or peptide which binds the peptide, such as the FLAG tag.

Suitable host cells for expression of Hu-Asp polypeptides includes prokaryotes, yeast, and higher eukaryotic cells. Suitable prokaryotic hosts to be used for the expression of Hu-Asp include bacteria of the genera *Escherichia*, *Bacillus*, and *Salmonella*, as well as members of the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. For expression in, *e.g.*, *E. coli*, a Hu-Asp polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in a prokaryotic host. The N-terminal Met may optionally then be cleaved from the expressed Hu-Asp polypeptide. Other N-terminal amino acid residues can be added to the Hu-Asp polypeptide to facilitate expression in *Escherichia coli* including but not limited to the T7 leader sequence, the T7-caspase 8

leader sequence, as well as others leaders including tags for purification such as the 6-His tag (Example 9). Hu-Asp polypeptides expressed in *E. coli* may be shortened by removal of the cytoplasmic tail, the transmembrane domain, or the membrane proximal region. Hu-Asp polypeptides expressed in *E. coli* may be obtained in either  
5 a soluble form or as an insoluble form which may or may not be present as an inclusion body. The insoluble polypeptide may be rendered soluble by guanidine HCl, urea or other protein denaturants, then refolded into a soluble form before or after purification by dilution or dialysis into a suitable aqueous buffer. If the inactive proform of the Hu-Asp was produced using recombinant methods, it may be rendered  
10 active by cleaving off the prosegment with a second suitable protease such as human immunodeficiency virus protease.

Expression vectors for use in prokaryotic hosts generally comprises one or more phenotypic selectable marker genes. Such genes generally encode, *e.g.*, a protein that confers antibiotic resistance or that supplies an auxotrophic requirement.  
15 A wide variety of such vectors are readily available from commercial sources. Examples include pSPORT vectors, pGEM vectors (Promega), pPROEX vectors (LTI, Bethesda, MD), Bluescript vectors (Stratagene), pET vectors (Novagen) and pQE vectors (Qiagen).

Hu-Asp may also be expressed in yeast host cells from genera including  
20 *Saccharomyces*, *Pichia*, and *Kluveromyces*. Preferred yeast hosts are *S. cerevisiae* and *P. pastoris*. Yeast vectors will often contain an origin of replication sequence from a 2T yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Vectors replicable in both yeast and *E. coli* (termed shuttle  
25 vectors) may also be used. In addition to the above-mentioned features of yeast vectors, a shuttle vector will also include sequences for replication and selection in *E. coli*. Direct secretion of Hu-Asp polypeptides expressed in yeast hosts may be accomplished by the inclusion of nucleotide sequence encoding the yeast I-factor leader sequence at the 5' end of the Hu-Asp-encoding nucleotide sequence.

Insect host cell culture systems may also be used for the expression of Hu-Asp polypeptides. In a preferred embodiment, the Hu-Asp polypeptides of the invention are expressed using an insect cell expression system (*see* Example 10). Additionally, a baculovirus expression system can be used for expression in insect cells as reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988).

In another preferred embodiment, the Hu-Asp polypeptide is expressed in mammalian host cells. Nonlimiting examples of suitable mammalian cell lines include the COS7 line of monkey kidney cells (Gluzman *et al.*, *Cell* 23:175 (1981)), human embryonic kidney cell line 293, and Chinese hamster ovary (CHO) cells. Preferably, Chinese hamster ovary (CHO) cells are used for expression of Hu-Asp proteins (Example 11).

The choice of a suitable expression vector for expression of the Hu-Asp polypeptides of the invention will of course depend upon the specific mammalian host cell to be used, and is within the skill of the ordinary artisan. Examples of suitable expression vectors include pcDNA3 (Invitrogen) and pSVL (Pharmacia Biotech). A preferred vector for expression of Hu-Asp polypeptides is pcDNA3.1-Hygro (Invitrogen). Expression vectors for use in mammalian host cells may include transcriptional and translational control sequences derived from viral genomes. Commonly used promoter sequences and enhancer sequences which may be used in the present invention include, but are not limited to, those derived from human cytomegalovirus (CMV), Adenovirus 2, Polyoma virus, and Simian virus 40 (SV40). Methods for the construction of mammalian expression vectors are disclosed, for example, in Okayama and Berg (*Mol. Cell. Biol.* 3:280 (1983)); Cosman *et al.* (*Mol. Immunol.* 23:935 (1986)); Cosman *et al.* (*Nature* 312:768 (1984)); EP-A-0367566; and WO 91/18982.

The polypeptides of the present invention may also be used to raise polyclonal and monoclonal antibodies, which are useful in diagnostic assays for detecting Hu-Asp polypeptide expression. Such antibodies may be prepared by conventional techniques. See, for example, *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1988);

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mutation found in a Swedish kindred with inherited AD in which KM is changed to NL, such peptide comprising the sequence SEVNLDAEFR (SEQ ID NO: 63) in an acidic buffering solution, preferably an acidic buffering solution of pH5.5 (see Example 12) using cleavage of the peptide monitored by high performance liquid chromatography as a measure of Hu-Asp proteolytic activity. Preferred assays for proteolytic activity utilize internally quenched peptide assay substrates. Such suitable substrates include peptides which have attached a paired flurophore and quencher including but not limited to 7-amino-4-methyl coumarin and dinitrophenol, respectively, such that cleavage of the peptide by the Hu-Asp results in increased fluorescence due to physical separation of the flurophore and quencher. Other paired flurophores and quenchers include bodipy-tetramethylrhodamine and QSY-5 (Molecular Probes, Inc.). In a variant of this assay, biotin or another suitable tag may be placed on one end of the peptide to anchor the peptide to a substrate assay plate and a flurophore may be placed at the other end of the peptide. Useful flurophores include those listed above as well as Europium labels such as W8044 (EG&g Wallac, Inc.). Cleavage of the peptide by Asp2 will release the flurophore or other tag from the plate, allowing compounds to be assayed for inhibition of Asp2 proteolytic cleavage as shown by an increase in retained fluorescence. Preferred colorimetric assays of Hu-Asp proteolytic activity utilize other suitable substrates that include the P2 and P1 amino acids comprising the recognition site for cleavage linked to o-nitrophenol through an amide linkage, such that cleavage by the Hu-Asp results in an increase in optical density after altering the assay buffer to alkaline pH.

In another embodiment, the invention relates to a method for the identification of an agent that increases the activity of a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method comprising

- (a) determining the activity of said Hu-Asp polypeptide in the presence of a test agent and in the absence of a test agent; and
- (b) comparing the activity of said Hu-Asp polypeptide determined in the presence of said test agent to the activity of said Hu-Asp polypeptide determined in the absence of said test agent;

whereby a higher level of activity in the presence of said test agent than in the absence of said test agent indicates that said test agent has increased the activity of said Hu-Asp polypeptide. Such tests can be performed with Hu-Asp polypeptide in a cell free system and with cultured cells that express Hu-Asp as well as variants or isoforms thereof.

In another embodiment, the invention relates to a method for the identification of an agent that decreases the activity of a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method comprising

- (a) determining the activity of said Hu-Asp polypeptide in the presence of a test agent and in the absence of a test agent; and

- (b) comparing the activity of said Hu-Asp polypeptide determined in the presence of said test agent to the activity of said Hu-Asp polypeptide determined in the absence of said test agent;

whereby a lower level of activity in the presence of said test agent than in the absence of said test agent indicates that said test agent has decreased the activity of said Hu-Asp polypeptide. Such tests can be performed with Hu-Asp polypeptide in a cell free system and with cultured cells that express Hu-Asp as well as variants or isoforms thereof.

In another embodiment, the invention relates to a novel cell line (HEK125.3 cells) for measuring processing of amyloid  $\beta$  peptide ( $A\beta$ ) from the amyloid protein precursor (APP). The cells are stable transformants of human embryonic kidney 293 cells (HEK293) with a bicistronic vector derived from pIRES-EGFP (Clontech) containing a modified human APP cDNA, an internal ribosome entry site and an enhanced green fluorescent protein (EGFP) cDNA in the second cistron. The APP cDNA was modified by adding two lysine codons to the carboxyl terminus of the APP coding sequence. This increases processing of  $A\beta$  peptide from human APP by 2-4 fold. This level of  $A\beta$  peptide processing is 60 fold higher than is seen in nontransformed HEK293 cells. HEK125.3 cells will be useful for assays of compounds that inhibit  $A\beta$  peptide processing. This invention also includes addition of two lysine residues to the C-terminus of other APP isoforms including the 751 and

770 amino acid isoforms, to isoforms of APP having mutations found in human AD including the Swedish KM-NL and V717-F mutations, to C-terminal fragments of APP, such as those beginning with the  $\beta$ -secretase cleavage site, to C-terminal fragments of APP containing the  $\beta$ -secretase cleavage site which have been operably  
 5 linked to an N-terminal signal peptide for membrane insertion and secretion, and to C-terminal fragments of APP which have been operably linked to an N-terminal signal peptide for membrane insertion and secretion and a reporter sequence including but not limited to green fluorescent protein or alkaline phosphatase, such that  $\beta$ -secretase cleavage releases the reporter protein from the surface of cells expressing  
 10 the polypeptide.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

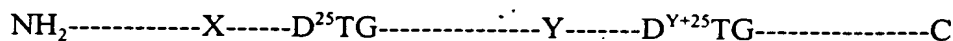
15

### Example 1

#### Development of a Search Algorithm Useful for the Identification of Aspartyl Proteases, and Identification of *C. elegans* Aspartyl Protease Genes in Wormpep 12

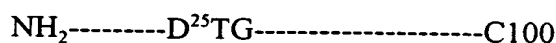
##### *Materials and Methods:*

20 Classical aspartyl proteases such as pepsin and renin possess a two-domain structure which folds to bring two aspartyl residues into proximity within the active site. These are embedded in the short tripeptide motif DTG, or more rarely, DSG. The DTG or DSG active site motif appears at about residue 25-30 in the enzyme, but at about 65-70 in the proenzyme (prorenin, pepsinogen). This motif appears again about  
 25 150-200 residues downstream. The proenzyme is activated by cleavage of the N-terminal prodomain. This pattern exemplifies the double domain structure of the modern day aspartyl enzymes which apparently arose by gene duplication and divergence. Thus;



30 where X denotes the beginning of the enzyme, following the N-terminal prodomain, and Y denotes the center of the molecule where the gene repeat begins again.

In the case of the retroviral enzymes such as the HIV protease, they represent only a half of the two-domain structures of well-known enzymes like pepsin, cathepsin D, renin, etc. They have no prosegment, but are carved out of a polyprotein precursor containing the *gag* and *pol* proteins of the virus. They can be represented by:



This "monomer" only has about 100 aa, so is extremely parsimonious as compared to the other aspartyl protease "dimers" which have of the order of 330 or so aa, not counting the N-terminal prodomain.

The limited length of the eukaryotic aspartyl protease active site motif makes it difficult to search EST collections for novel sequences. EST sequences typically average 250 nucleotides, and so in this case would be unlikely to span both aspartyl protease active site motifs. Instead, we turned to the *C. elegans* genome. The *C. elegans* genome is estimated to contain around 13,000 genes. Of these, roughly 12,000 have been sequenced and the corresponding hypothetical open reading frame (ORF) has been placed in the database Wormpep12. We used this database as the basis for a whole genome scan of a higher eukaryote for novel aspartyl proteases, using an algorithm that we developed specifically for this purpose. The following AWK script for locating proteins containing two DTG or DSG motifs was used for the search, which was repeated four times to recover all pairwise combinations of the aspartyl motif.

```

BEGIN{RS=">"}           /* defines ">" as record separator for FASTA format */
{
pos = index($0,"DTG")    /* finds "DTG" in record */
if (pos>0) {
    rest = substr($0,pos+3) /* get rest of record after first DTG */
    pos2 = index(rest,"DTG") /* find second DTG */
    if (pos2>0) printf ("%s%s\n", ">", $0) /* report hits */
}
}

```

The AWK script shown above was used to search Wormpep12, which was downloaded from <ftp.sanger.ac.uk/pub/databases/wormpep>, for sequence entries



Wormpep12 failed to reveal additional candidate aspartyl proteases in the *C. elegans* genome containing two repeats of the DTG or DSG motif.

BLASTX search with each *C. elegans* sequence against SWISS-PROT, GenPep and TREMBL revealed that R12H7.2 was the closest worm homologue to the known mammalian aspartyl proteases, and that T18H9.2 was somewhat more  
5 distantly related, while CEASP1, F21F8.3, F21F8.4, and F21F8.7 formed a subcluster which had the least sequence homology to the mammalian sequences.

**Discussion:**

APP, the presenilins, and p35, the activator of cdk5, all undergo intracellular proteolytic processing at sites which conform to the substrate specificity of the HIV protease. Dysregulation of a cellular aspartyl protease with the same substrate specificity, might therefore provide a unifying mechanism for causation of the plaque and tangle pathologies in AD. Therefore, we sought to identify novel human aspartyl proteases. A whole genome scan in *C. elegans* identified seven open reading frames that adhere to the aspartyl protease profile that we had identified. These seven aspartyl proteases probably comprise the complete complement of such proteases in a simple, multicellular eukaryote. These include four closely related aspartyl proteases unique to *C. elegans* which probably arose by duplication of an ancestral gene. The other three candidate aspartyl proteases (T18H9.2, R12H7.2 and C11D2.2) were found to have homology to mammalian gene sequences.

### Example 2

# Identification of Novel Human Aspartyl Proteases Using Database Mining by Genome Bridging

### Materials and Methods:

**Computer-assisted analysis of EST databases, cDNA , and predicted polypeptide sequences:**

Exhaustive homology searches of EST databases with the CEASP1, F21F8.3, F21F8.4, and F21F8.7 sequences failed to reveal any novel mammalian homologues. TBLASTN searches with R12H7.2 showed homology to cathepsin D, cathepsin E,

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### *Full-length cDNA cloning of Hu-Asp1*

The open reading frame of *C. elegans* gene T18H9.2CE was used to query Incyte LifeSeq and LifeSeq-FL databases and a single electronic assembly referred to as 1863920CE1 was detected. The 5' most cDNA clone in this contig, 1863920, was obtained from Incyte and completely sequenced on both strands. Translation of the open reading frame contained within clone 1863920 revealed the presence of the duplicated aspartyl protease active site motif (DTG/DSG) but the 5' end was incomplete. The remainder of the Hu-Asp1 coding sequence was determined by 5' Marathon RACE analysis using a human placenta Marathon ready cDNA template (Clontech). A 3'-antisense oligonucleotide primer specific for the 5' end of clone 1863920 was paired with the 5'-sense primer specific for the Marathon ready cDNA synthetic adaptor in the PCR. Specific PCR products were directly sequenced by cycle sequencing and the resulting sequence assembled with the sequence of clone 1863920 to yield the complete coding sequence of Hu-Asp-1 (SEQ ID No. 1).

Several interesting features are present in the primary amino acid sequence of Hu-Asp1 (Figure 1, SEQ ID No. 2). The sequence contains a signal peptide (residues 1-20 in SEQ ID No. 2), a pro-segment, and a catalytic domain containing two copies of the aspartyl protease active site motif (DTG/DSG). The spacing between the first and second active site motifs is about 200 residues which should correspond to the expected size of a single, eukaryotic aspartyl protease domain. More interestingly, the sequence contains a predicted transmembrane domain (residues 469-492 in SEQ ID No.2) near its C-terminus which suggests that the protease is anchored in the membrane. This feature is not found in any other aspartyl protease.

### *Cloning of a full-length Hu-Asp-2 cDNAs:*

As is described above in Example 1, genome wide scan of the *Caenorhabditis elegans* database WormPep12 for putative aspartyl proteases and subsequent mining of human EST databases revealed a human ortholog to the *C. elegans* gene T18H9.2 referred to as Hu-Asp1. The assembled contig for Hu-Asp1 was used to query for human paralogs using the BLAST search tool in human EST databases and a single



significant match (2696295CE1) with approximately 60% shared identity was found in the LifeSeq FL database. Similar queries of either gb105PubEST or the family of human databases available from TIGR did not identify similar EST clones. cDNA clone 2696295, identified by single pass sequence analysis from a human uterus cDNA library, was obtained from Incyte and completely sequence on both strands. This clone contained an incomplete 1266 bp open-reading frame that encoded a 422 amino acid polypeptide but lacked an initiator ATG on the 5' end. Inspection of the predicted sequence revealed the presence of the duplicated aspartyl protease active site motif DTG/DSG, separated by 194 amino acid residues. Subsequent queries of later releases of the LifeSeq EST database identified an additional ESTs, sequenced from a human astrocyte cDNA library (4386993), that appeared to contain additional 5' sequence relative to clone 2696295. Clone 4386993 was obtained from Incyte and completely sequenced on both strands. Comparative analysis of clone 4386993 and clone 2696295 confirmed that clone 4386993 extended the open-reading frame by 31 amino acid residues including two in-frame translation initiation codons. Despite the presence of the two in-frame ATGs, no in-frame stop codon was observed upstream of the ATG indicating that the 4386993 may not be full-length. Furthermore, alignment of the sequences of clones 2696295 and 4386993 revealed a 75 base pair insertion in clone 2696295 relative to clone 4386993 that results in the insertion of 25 additional amino acid residues in 2696295. The remainder of the Hu-Asp2 coding sequence was determined by 5' Marathon RACE analysis using a human hippocampus Marathon ready cDNA template (Clontech). A 3'-antisense oligonucleotide primer specific for the shared 5'-region of clones 2696295 and 4386993 was paired with the 5'-sense primer specific for the Marathon ready cDNA synthetic adaptor in the PCR. Specific PCR products were directly sequenced by cycle sequencing and the resulting sequence assembled with the sequence of clones 2696295 and 4386993 to yield the complete coding sequence of Hu-Asp2(a) (SEQ ID No. 3) and Hu-Asp2(b) (SEQ ID No. 5), respectively.

Several interesting features are present in the primary amino acid sequence of Hu-Asp2(a) (Figure 2 and SEQ ID No. 4) and Hu-Asp-2(b) (Figure 3, SEQ ID No. 6).

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this region of murine Asp2 were then synthesized and used to amplify regions of the murine gene. Murine genomic DNA, derived from strain 129/SvJ, was amplified in the PCR (25 cycles) using various primer sets specific for murine Asp2 and the products analyzed by agarose gel electrophoresis. The primer set Zoo-1 and Zoo-4 amplified a 750 bp fragment that contained approximately 600 bp of intron sequence based on comparison to the known cDNA sequence. This primer set was then used to screen a murine BAC library by PCR, a single genomic clone was isolated and this cloned was confirmed contain the murine Asp2 gene by DNA sequence analysis. Shotgun DNA sequencing of this Asp2 genomic clone and comparison to the cDNA sequences of both Hu-Asp2 and the partial murine cDNA sequences defined the full-length sequence of murine Asp2 (SEQ ID No. 7). The predicted amino acid sequence of murine Asp2 (SEQ ID No. 8) showed 96.4% shared identity (GCG BestFit algorithm) with 18/501 amino acid residue substitutions compared to the human sequence (Figure 4). The proteolytic processing of murine Asp2(a) is believed to be analogous to the processing described above for human Asp2(a). In addition, a variant lacking amino acid residues 190-214 of SEQ ID NO: 8 is specifically contemplated as a murine Asp2(b) polypeptide. All forms of murine Asp2(b) gene and protein are intended as aspects of the invention.

### Example 4

### Tissue Distribution of Expression of Hu-Asp2 Transcripts

### Materials and Methods:

The tissue distribution of expression of Hu-Asp-2 was determined using multiple tissue Northern blots obtained from Clontech (Palo Alto, CA). Incyte clone 2696295 in the vector pINCY was digested to completion with *EcoRI/NotI* and the 1.8 kb cDNA insert purified by preparative agarose gel electrophoresis. This fragment was radiolabeled to a specific activity  $> 1 \times 10^9$  dpm/ $\mu$ g by random priming in the presence of [ $\alpha$ - $^{32}$ P-dATP] ( $>3000$  Ci/mmol, Amersham, Arlington Heights, IL) and Klenow fragment of DNA polymerase I. Nylon filters containing denatured, size fractionated poly A<sup>+</sup> RNAs isolated from different human tissues were hybridized

with  $2 \times 10^6$  dpm/ml probe in ExpressHyb buffer (Clontech, Palo Alto, CA) for 1 hour at 68 °C and washed as recommended by the manufacture. Hybridization signals were visualized by autoradiography using BioMax XR film (Kodak, Rochester, NY) with intensifying screens at -80 °C.

### Results and Discussion:

Limited information on the tissue distribution of expression of Hu-Asp-2 transcripts was obtained from database analysis due to the relatively small number of ESTs detected using the methods described above ( $< 5$ ). In an effort to gain further information on the expression of the Hu-Asp2 gene, Northern analysis was employed to determine both the size(s) and abundance of Hu-Asp2 transcripts. PolyA<sup>+</sup> RNAs isolated from a series of peripheral tissues and brain regions were displayed on a solid support following separation under denaturing conditions and Hu-Asp2 transcripts were visualized by high stringency hybridization to radiolabeled insert from clone 2696295. The 2696295 cDNA probe visualized a constellation of transcripts that migrated with apparent sizes of 3.0kb, 4.4 kb and 8.0 kb with the latter two transcript being the most abundant.

Across the tissues surveyed, Hu-Asp2 transcripts were most abundant in pancreas and brain with lower but detectable levels observed in all other tissues examined except thymus and PBLs. Given the relative abundance of Hu-Asp2 transcripts in brain, the regional expression in brain regions was also established. A similar constellation of transcript sizes were detected in all brain regions examined [cerebellum, cerebral cortex, occipital pole, frontal lobe, temporal lobe and putamen] with the highest abundance in the medulla and spinal cord.

### Example 5

### Northern Blot Detection of HuAsp-1 and HuAsp-2 Transcripts in Human Cell Lines

A variety of human cell lines were tested for their ability to produce Hu-Asp1 and Asp2 mRNA. Human embryonic kidney (HEK-293) cells, African green monkey (Cos-7) cells, Chinese hamster ovary (CHO) cells, HELA cells, and the

neuroblastoma cell line IMR-32 were all obtained from the ATCC. Cells were cultured in DME containing 10% FCS except CHO cells which were maintained in  $\alpha$ -MEM/10% FCS at 37 °C in 5% CO<sub>2</sub> until they were near confluence. Washed monolayers of cells (3 X 10<sup>7</sup>) were lysed on the dishes and poly A<sup>+</sup> RNA extracted using the Qiagen Oligotex Direct mRNA kit. Samples containing 2  $\mu$ g of poly A<sup>+</sup> RNA from each cell line were fractionated under denaturing conditions (glyoxal-treated), transferred to a solid nylon membrane support by capillary action, and transcripts visualized by hybridization with random-primed labeled (<sup>32</sup>P) coding sequence probes derived from either Hu-Asp1 or Hu-Asp2. Radioactive signals were detected by exposure to X-ray film and by image analysis with a PhosphorImager.

The Hu-Asp1 cDNA probe visualized a similar constellation of transcripts (2.6 kb and 3.5 kb) that were previously detected in human tissues. The relative abundance determined by quantification of the radioactive signal was Cos-7 > HEK 292 = HELA > IMR32.

The Hu-Asp2 cDNA probe also visualized a similar constellation of transcripts compared to tissue (3.0 kb, 4.4 kb, and 8.0 kb) with the following relative abundance; HEK 293 > Cos 7 > IMR32 > HELA.

#### Example 6

##### Modification of APP to increase A $\beta$ processing for in vitro screening

Human cell lines that process A $\beta$  peptide from APP provide a means to screen in cellular assays for inhibitors of  $\beta$ - and  $\gamma$ -secretase. Production and release of A $\beta$  peptide into the culture supernatant is monitored by an enzyme-linked immunosorbent assay (EIA). Although expression of APP is widespread and both neural and non-neuronal cell lines process and release A $\beta$  peptide, levels of endogenous APP processing are low and difficult to detect by EIA. A $\beta$  processing can be increased by expressing in transformed cell lines mutations of APP that enhance A $\beta$  processing. We made the serendipitous observation that addition of two lysine residues to the carboxyl terminus of APP695 increases A $\beta$  processing still further. This allowed us

to create a transformed cell line that releases A $\beta$  peptide into the culture medium at the remarkable level of 20,000 pg/ml.

### ***Materials And Methods***

#### ***Materials:***

5 Human embryonic kidney cell line 293 (HEK293 cells) were obtained internally. The vector pIRES-EGFP was purchased from Clontech. Oligonucleotides for mutation using the polymerase chain reaction (PCR) were purchased from Genosys. A plasmid containing human APP695 (SEQ ID No. 9 [nucleotide] and SEQ ID No. 10 [amino acid]) was obtained from Northwestern University Medical School.  
10 This was subcloned into pSK (Stratagene) at the *Not*I site creating the plasmid pAPP695.

#### ***Mutagenesis protocol:***

The Swedish mutation (K670N, M671L) was introduced into pAPP695 using the Stratagene Quick Change Mutagenesis Kit to create the plasmid pAPP695NL  
15 (SEQ ID No. 11 [nucleotide] and SEQ ID No. 12 [amino acid]). To introduce a di-lysine motif at the C-terminus of APP695, the forward primer #276 5' GACTGACCACTCGACCAGGTTC (SEQ ID No. 47) was used with the "patch" primer #274 5'  
CGAATTAAATTCCAGCACACTGGCTACTTCTTGTTCTGCATCTCAAAGAAC  
20 (SEQ ID No. 48) and the flanking primer #275 CGAATTAAATTCCAGCACACTGGCTA (SEQ ID No. 49) to modify the 3' end of the APP695 cDNA (SEQ ID No. 15 [nucleotide] and SEQ ID No. 16 [amino acid]). This also added a BstX1 restriction site that will be compatible with the BstX1 site in the multiple cloning site of pIRES-EGFP. PCR amplification was performed with a  
25 Clontech HF Advantage cDNA PCR kit using the polymerase mix and buffers supplied by the manufacturer. For "patch" PCR, the patch primer was used at 1/20th the molar concentration of the flanking primers. PCR amplification products were purified using a QIAquick PCR purification kit (Qiagen). After digestion with restriction enzymes, products were separated on 0.8% agarose gels and then excised  
30 DNA fragments were purified using a QIAquick gel extraction kit (Qiagen).

To reassemble a modified APP695-Sw cDNA, the 5' Not1-Bgl2 fragment of the APP695-Sw cDNA and the 3' Bgl2-BstX1 APP695 cDNA fragment obtained by PCR were ligated into pIRES-EGFP plasmid DNA opened at the Not1 and BstX1 sites. Ligations were performed for 5 minutes at room temperature using a Rapid DNA Ligation kit (Boehringer Mannheim) and transformed into Library Efficiency DH5a Competent Cells (GibcoBRL Life Technologies). Bacterial colonies were screened for inserts by PCR amplification using primers #276 and #275. Plasmid DNA was purified for mammalian cell transfection using a QIAprep Spin Miniprep kit (Qiagen). The construct obtained was designated pMG125.3 (APPSW-KK, SEQ ID No. 17 [nucleotide] and SEQ ID No. 18 [amino acid]).

#### Mammalian Cell Transfection:

HEK293 cells for transfection were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Cotransfections were performed using LipofectAmine (Gibco-BRL) with 3 µg pMG125.3 DNA and 9 µg pcDNA3.1 DNA per  $10 \times 10^6$  cells. Three days posttransfection, cells were passaged into medium containing G418 at a concentration of 400 µg/ml. After three days growth in selective medium, cells were sorted by their fluorescence.

#### Clonal Selection of 125.3 cells by FACS:

Cell samples were analyzed on an EPICS Elite ESP flow cytometer (Coulter, Hialeah, FL) equipped with a 488 nm excitation line supplied by an air-cooled argon laser. EGFP emission was measured through a 525 nm band-pass filter and fluorescence intensity was displayed on a 4-decade log scale after gating on viable cells as determined by forward and right angle light scatter. Single green cells were separated into each well of one 96 well plate containing growth medium without G418. After a four day recovery period, G418 was added to the medium to a final concentration of 400 µg/ml. After selection, 32% of the wells contained expanding clones. Wells with clones were expanded from the 96 well plate to a 24 well plate and then a 6 well plate with the fastest growing colonies chosen for expansion at each passage. The final cell line selected was the fastest growing of the final six passaged. This clone, designated 125.3, has been maintained in G418 at 400 ug/ml with passage

every four days into fresh medium. No loss of A $\beta$  production or EGFP fluorescence has been seen over 23 passages.

*A $\beta$  EIA Analysis (Double Antibody Sandwich ELISA for hA $\beta$  1-40/42):*

Cell culture supernatants harvested 48 hours after transfection were analyzed in a standard A $\beta$  EIA as follows. Human A $\beta$  1-40 or 1-42 was measured using monoclonal antibody (mAb) 6E10 (Senetek, St. Louis, MO) and biotinylated rabbit antiserum 162 or 164 (New York State Institute for Basic Research, Staten Island, NY) in a double antibody sandwich ELISA. The capture antibody 6E10 is specific to an epitope present on the N-terminal amino acid residues 1-16 of hA $\beta$ . The conjugated detecting antibodies 162 and 164 are specific for hA $\beta$  1-40 and 1-42, respectively. Briefly, a Nunc Maxisorp 96 well immunoplate was coated with 100  $\mu$ l/well of mAb 6E10 (5 $\mu$ g/ml) diluted in 0.1M carbonate-bicarbonate buffer, pH 9.6 and incubated at 4°C overnight. After washing the plate 3x with 0.01M DPBS (Modified Dulbecco's Phosphate Buffered Saline (0.008M sodium phosphate, 0.002M potassium phosphate, 0.14M sodium chloride, 0.01 M potassium chloride, pH 7.4) from Pierce, Rockford, IL) containing 0.05% of Tween-20 (DPBST), the plate was blocked for 60 minutes with 200  $\mu$ l of 10% normal sheep serum (Sigma) in 0.01M DPBS to avoid non-specific binding. Human A $\beta$  1-40 or 1-42 standards 100  $\mu$ l/well (Bachem, Torrance, CA) diluted, from a 1mg/ml stock solution in DMSO, in culture medium was added after washing the plate, as well as 100  $\mu$ l/well of sample, *e.g.*, conditioned medium of transfected cells.

The plate was incubated for 2 hours at room temperature and 4°C overnight. The next day, after washing the plate, 100  $\mu$ l/well biotinylated rabbit antiserum 162 1:400 or 164 1:50 diluted in DPBST + 0.5% BSA was added and incubated at room temperature for 1 hour, 15 minutes. Following washes, 100  $\mu$ l/well neutravidin-horseradish peroxidase (Pierce, Rockford, IL) diluted 1:10,000 in DPBST was applied and incubated for 1 hour at room temperature. After the last washes 100  $\mu$ l/well of o-phenyldiamine dihydrochloride (Sigma Chemicals, St. Louis, MO) in 50mM citric acid/100mM sodium phosphate buffer (Sigma Chemicals, St. Louis, MO), pH 5.0, was added as substrate and the color development was monitored at



450nm in a kinetic microplate reader for 20 minutes using Soft max Pro software. All standards and samples were run in triplicates. The samples with absorbance values falling within the standard curve were extrapolated from the standard curves using Soft max Pro software and expressed in pg/ml culture medium.

5 *Results:*

Addition of two lysine residues to the carboxyl terminus of APP695 greatly increases A $\beta$  processing in HEK293 cells as shown by transient expression (Table 1). Addition of the di-lysine motif to APP695 increases A $\beta$  processing to that seen with the APP695 containing the Swedish mutation. Combining the di-lysine motif with the  
10 Swedish mutation further increases processing by an additional 2.8 fold.

Cotransformation of HEK293 cells with pMG125.3 and pcDNA3.1 allowed dual selection of transformed cells for G418 resistance and high level expression of EGFP. After clonal selection by FACS, the cell line obtained, produces a remarkable  
15 20,000 pg A $\beta$  peptide per ml of culture medium after growth for 36 hours in 24 well plates. Production of A $\beta$  peptide under various growth conditions is summarized in Table 2.

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**TABLE 1**

Release of A $\beta$  peptide into the culture medium 48 hours after transient transfection of HEK293 cells with the indicated vectors containing wildtype or modified APP. Values tabulated are mean + SD and P-value for pairwise comparison using Student's t-test assuming unequal variances.

APP Construct	A $\beta$ 1-40 peptide (pg/ml)	Fold Increase	P-value
pIRES-EGFP vector	147 + 28	1.0	
wt APP695 (142.3)	194 + 15	1.3	0.051
wt APP695-KK (124.1)	424 + 34	2.8	3 x 10 <sup>-5</sup>
APP695-Sw (143.3)	457 + 65	3.1	2 x 10 <sup>-3</sup>
APP695-SwKK (125.3)	1308 + 98	8.9	3 x 10 <sup>-4</sup>

**TABLE 2**

Release of A $\beta$  peptide from HEK125.3 cells under various growth conditions.

5	Type of Culture Plate	Volume of Medium	Duration of Culture	A $\beta$ 1-40 (pg/ml)	A $\beta$ 1-42 (pg/ml)
10	24 well plate	400 ul	36 hr	28,036	1,439

**Example 7**

**Antisense oligomer inhibition of Abeta processing in HEK125.3 cells**

15       The sequences of Hu-Asp1 and Hu-Asp2 were provided to Sequitur, Inc  
(Natick, MA) for selection of targeted sequences and design of 2nd generation  
chimeric antisense oligomers using proprietary technology (Sequitur Ver. D Pat  
pending #3002). Antisense oligomers Lot# S644, S645, S646 and S647 were targeted  
against Asp1. Antisense oligomers Lot# S648, S649, S650 and S651 were targeted  
20       against Asp2. Control antisense oligomers Lot# S652, S653, S655, and S674 were  
targeted against an irrelevant gene and antisense oligomers Lot #S656, S657, S658,  
and S659 were targeted against a second irrelevant gene.

For transfection with the antisense oligomers, HEK125.3 cells were grown to  
about 50% confluence in 6 well plates in Minimal Essential Medium (MEM)  
25       supplemented with 10% fetal calf serum. A stock solution of oligofectin G (Sequitur  
Inc., Natick, MA) at 2 mg/ml was diluted to 50  $\mu$ g/ml in serum free MEM.  
Separately, the antisense oligomer stock solution at 100  $\mu$ M was diluted to 800 nM in  
Opti-MEM (GIBCO-BRL, Grand Island, NY). The diluted stocks of oligofectin G  
and antisense oligomer were then mixed at a ratio of 1:1 and incubated at room  
30       temperature. After 15 minutes incubation, the reagent was diluted 10 fold into MEM

containing 10% fetal calf serum and 2 ml was added to each well of the 6 well plate after first removing the old medium. After transfection, cells were grown in the continual presence of the oligofectin G/antisense oligomer. To monitor A $\beta$  peptide release, 400  $\mu$ l of conditioned medium was removed periodically from the culture well and replaced with fresh medium beginning 24 hours after transfection. A $\beta$  peptides in the conditioned medium were assayed via immunoprecipitation and Western blotting. Data reported are from culture supernatants harvested 48 hours after transfection.

The 16 different antisense oligomers obtained from Sequitur Inc. were transfected separately into HEK125.3 cells to determine their affect on A $\beta$  peptide processing. Only antisense oligomers targeted against Asp2 significantly reduced Abeta processing by HEK125.3 cells. Both A $\beta$  (1-40) and A $\beta$  (1-42) were inhibited by the same degree. In Table 3, percent inhibition is calculated with respect to untransfected cells. Antisense oligomer reagents giving greater than 50% inhibition are marked with an asterisk. For ASP2, 4 of 4 antisense oligomers gave greater than 50% inhibition with an average inhibition of 62% for A $\beta$  1-40 processing and 60% for A $\beta$  1-42 processing.

[illegible]

5	Gene Targeted	Antisense Oligomer	Abeta (1-40)	Abeta (1-42)
10	Asp2-1	S648	71%*	67%*
	Asp2-2	S649	83%*	76%*
	Asp2-3	S650	46%*	50%*
	Asp2-4	S651	47%*	46%*
15	Con1-1	S652	13%	18%
	Con1-2	S653	35%	30%
	Con1-3	S655	9%	18%
	Con1-4	S674	29%	18%
20	Con2-1	S656	12%	18%
	Con2-2	S657	16%	19%
	Con2-3	S658	8%	35%
	Con2-4	S659	3%	18%
30				

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*J. NeuroSci. Res.*, (1994) 39: 482-93; and Asami-Odaka *et al.*, *Biochem.*, (1995) 34:10272-8.] Essentially identical results were obtained in the neuroblastoma cells as the HEK293 cells. As shown in Table 3B, the pair of Asp2 antisense oligomers reduced Asp2 mRNA by roughly one-half, while the pair of reverse control oligomers lacked this effect (Table 3B).

Table 3B

Reduction of A $\beta$ 40 and A $\beta$ 42 in human neuroblastoma IMR-32 cells and mouse neuroblastoma Neuro-2A cells treated with Asp2 antisense and control oligomers as indicated. Oligomers were transfected in quadruplicate cultures. Values tabulated are normalized against cultures treated with oligofectin-G<sup>TM</sup> only (mean + SD, \*\* p<0.001 compared to reverse control oligomer).

	Asp2 mRNA	IMR-32 cells		Neuro-2A cells	
		A $\beta$ 40	A $\beta$ 42	A $\beta$ 40	A $\beta$ 42
Asp2-1A	-75%	-49 + 2%**	-42 + 14%**	-70 + 7%**	-67 + 2%**
Asp2-1R	0.16	-0 + 3%	21.26	-9 + 15%	1.05
Asp2-2A	-39%	-43 + 3%**	-44 + 18%**	-61 + 12%**	-61 + 12%**
Asp2-2R	0.47	12.2	19.22	6.15	-8 + 10%

Together with the reduction in Asp2 mRNA there was a concomitant reduction in the release of A $\beta$ 40 and A $\beta$ 42 peptides into the conditioned medium. Thus, Asp2 functions directly or indirectly in a human kidney and a human neuroblastoma cell line to facilitate the processing of APP into A $\beta$  peptides. Molecular cloning of the mouse Asp2 cDNA revealed a high degree of homology to human (>96% amino acid identity, see Example 3), and indeed, complete nucleotide identity at the sites targeted by the Asp2-1A and Asp2-2A antisense oligomers. Similar results were obtained in mouse Neuro-2a cells engineered to express APP-Sw-KK. The Asp2 antisense

oligomers reduced release of A $\beta$  peptides into the medium while the reverse control oligomers did not (Table 3B). Thus, the three antisense experiments with HEK293, IMR-32 and Neuro-2a cells indicate that Asp2 acts directly or indirectly to facilitate A $\beta$  processing in both somatic and neural cell lines.

5

### Example 8

#### Demonstration of Hu-Asp2 $\beta$ -Secretase Activity in Cultured Cells

Several mutations in APP associated with early onset Alzheimer's disease have been shown to alter A $\beta$  peptide processing. These flank the – and C-terminal cleavage sites that release A $\beta$  from APP. These cleavage sites are referred to as the  $\beta$ -secretase and  $\gamma$ -secretase cleavage sites, respectively. Cleavage of APP at the  $\beta$ -secretase site creates a C-terminal fragment of APP containing 99 amino acids of 11,145 daltons molecular weight. The Swedish KM–NL mutation immediately upstream of the  $\beta$ -secretase cleavage site causes a general increase in production of both the 1-40 and 1-42 amino acid forms of A $\beta$  peptide. The London VF mutation (V717–F in the APP770 isoform) has little effect on total A $\beta$  peptide production, but appears to preferentially increase the percentage of the longer 1-42 amino acid form of A $\beta$  peptide by affecting the choice of  $\beta$ -secretase cleavage site used during APP processing. Thus, we sought to determine if these mutations altered the amount and type of A $\beta$  peptide produced by cultured cells cotransfected with a construct directing expression of Hu-Asp2.

Two experiments were performed which demonstrate Hu-Asp2  $\beta$ -secretase activity in cultured cells. In the first experiment, treatment of HEK125.3 cells with antisense oligomers directed against Hu-Asp2 transcripts as described in Example 7 was found to decrease the amount of the C-terminal fragment of APP created by  $\beta$ -secretase cleavage (CTF99) (Figure 9). This shows that Hu-Asp2 acts directly or indirectly to facilitate  $\beta$ -secretase cleavage. In the second experiment, increased expression of Hu-Asp2 in transfected mouse Neuro2A cells is shown to increase accumulation of the CTF99  $\beta$ -secretase cleavage fragment (Figure 10). This increase is seen most easily when a mutant APP-KK clone containing a C-terminal di-lysine

30

motif is used for transfection. A further increase is seen when Hu-Asp2 is cotransfected with APP-Sw-KK containing the Swedish mutation KM -NL. The Swedish mutation is known to increase cleavage of APP by the  $\beta$ -secretase.

A second set of experiments demonstrate Hu-Asp2 facilitates  $\gamma$ -secretase activity in cotransfection experiments with human embryonic kidney HEK293 cells. Cotransfection of Hu-Asp2 with an APP-KK clone greatly increases production and release of soluble A $\beta$ 1-40 and A $\beta$ 1-42 peptides from HEK293 cells. There is a proportionately greater increase in the release of A $\beta$ 1-42. A further increase in production of A $\beta$ 1-42 is seen when Hu-Asp2 is cotransfected with APP-VF (SEQ ID No. 13 [nucleotide] and SEQ ID No. 14 [amino acid]) or APP-VF-KK SEQ ID No. 19 [nucleotide] and SEQ ID No. 20 [amino acid]) clones containing the London mutation V717-F. The V717-F mutation is known to alter cleavage specificity of the APP  $\gamma$ -secretase such that the preference for cleavage at the A $\beta$ 42 site is increased. Thus, Asp2 acts directly or indirectly to facilitate  $\gamma$ -secretase processing of APP at the  $\beta$ 42 cleavage site.

## Materials

### APP Constructs used

APP-Sw: APP695 containing the Swedish KM--NL mutation (SEQ ID No. 11  
25 and No. 12 , wherein the lysine (K) at residue 595 of APP695 is changed to  
asparagine (N) and the methionine (M) at residue 596 of APP695 is changed to  
leucine (L).),

APP-VF: APP695 containing the London V-F mutation (SEQ ID Nos. 13 & 14) (Affected residue 717 of the APP770 isoform corresponds with residue 642 of the



APP695 isoform. Thus, APP-VF as set in SEQ ID NO: 14 comprises the APP695 sequence, wherein the valine (V) at residue 642 is changed to phenylalanine (F).)

APP-KK: APP695 containing a C-terminal KK motif (SEQ ID Nos. 15 & 16).

APP-VF-KK: APP695-VF containing a C-terminal KK motif (SEQ ID Nos. 19 & 20).

*Transfection of antisense oligomers or plasmid DNA constructs in HEK293 cells, HEK125.3 cells and Neuro-2A cells,*

OptiMEM was added to a total volume of 1 ml, distributed 200  $\mu$ l per well and incubated 3 hours. Care was taken to hold constant the ratios of the two plasmids used for cotransfection as well as the total amount of DNA used in the transfection. The transfection media was replaced with DMEM, 10%FBS, NaPyruvate, with antibiotic/antimycotic and the cells were incubated under normal conditions (37°C, 5% CO<sub>2</sub>) for 48 hours. The conditioned media were removed to polypropylene tubes and stored at -80°C until assayed for the content of A $\beta$ 1-40 and A $\beta$ 1-42 by EIA as described in the preceding examples. Transfection of antisense oligomers into HEK125.3 cells was as described in Example 7.

25

### *Preparation of cell extracts, Western blot protocol*

Cells were harvested after being transfected with plasmid DNA for about 60 hours. First, cells were transferred to 15-ml conical tube from the plate and

centrifuged at 1,500 rpm for 5 minutes to remove the medium. The cell pellets were washed once with PBS. We then lysed the cells with lysis buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 0.1 mM sodium vanadate and 1% NP-40). The lysed cell mixtures were centrifuged at 5000 rpm and the supernatant was stored at -20°C as the cell extracts. Equal amounts of extracts from HEK125.3 cells transfected with the Asp2 antisense oligomers and controls were precipitated with antibody 369 that recognizes the C-terminus of APP and then CTF99 was detected in the immunoprecipitate with antibody 6E10. The experiment was repeated using C8, a second precipitating antibody that also recognizes the C-terminus of APP. For Western blot of extracts from mouse Neuro-2a cells cotransfected with Hu-Asp2 and APP-KK, APP-Sw-KK, APP-VF-KK or APP-VF, equal amounts of cell extracts were electrophoresed through 4-10% or 10-20% Tricine gradient gels (NOVEX, San Diego, CA). Full length APP and the CTF99  $\beta$ -secretase product were detected with antibody 6E10.

## Results

Transfection of HEK125.3 cells with Asp2-1 or Asp2-2 antisense oligomers reduces production of the CTF  $\beta$ -secretase product in comparison to cells similarly transfected with control oligomers having the reverse sequence (Asp2-1 reverse & Asp2-2 reverse), see Figure 9. Correspondingly, cotransfection of Hu-Asp2 into mouse Neuro-2a cells with the APP-KK construct increased the formation of CTF99. (See Fig. 10.) This was further increased if Hu-Asp2 was coexpressed with APP-Sw-KK, a mutant form of APP containing the Swedish KM-NL mutation that increases  $\beta$ -secretase processing.

Effects of Asp2 on the production of Ab peptides from endogenously expressed APP isoforms were assessed in HEK293 cells transfected with a construct expressing Asp2 or with the empty vector after selection of transformants with the antibiotic G418. A $\beta$ 40 production was increased in cells transformed with the Asp2 construct in comparison to those transformed with the empty vector DNA. A $\beta$ 40 levels in conditioned medium collected from the Asp2 transformed and control cultures was  $424 \pm 45$  pg/ml and  $113 \pm 58$  pg/ml, respectively ( $p < 0.001$ ). A $\beta$ 42

release was below the limit of detection by the EIA, while the release of sAPP $\alpha$  was unaffected,  $112 \pm 8$  ng/ml versus  $111 \pm 40$  ng/ml. This further indicates that Asp2 acts directly or indirectly to facilitate the processing and release of A $\beta$  from endogenously expressed APP.

5 Co-transfection of Hu-Asp2 with APP has little effect on A $\beta$ 40 production but increases A $\beta$ 42 production above background (Table 4). Addition of the di-lysine motif to the C-terminus of APP increases A $\beta$  peptide processing about two fold, although A $\beta$ 40 and A $\beta$ 42 production remain quite low (352 pg/ml and 21 pg/ml, respectively). Cotransfection of Asp2 with APP-KK further increases both A $\beta$ 40 and  
10 A $\beta$ 42 production.

The APP V717-F mutation has been shown to increase  $\gamma$ -secretase processing at the A $\beta$ 42 cleavage site. Cotransfection of Hu-Asp2 with the APP-VF or APP-VF-KK constructs increased A $\beta$ 42 production (a two fold increase with APP-VF and a four-fold increase with APP-VF-KK, Table 4), but had mixed effects on A $\beta$ 40  
15 production (a slight decrease with APP-VF, and a two fold increase with APP-VF-KK in comparison to the pcDNA cotransfection control. Thus, the effect of Asp2 on A $\beta$ 42 production was proportionately greater leading to an increase in the ratio of A $\beta$ 42/total Ab. Indeed, the ratio of A $\beta$ 42/total A $\beta$  reaches a very high value of 42% in HEK293 cells cotransfected with Hu-Asp2 and APP-VF-KK.  
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**Table 4**

Results of cotransfecting Hu-Asp2 or pcDNA plasmid DNA with various APP constructs containing the V717-F mutation that modifies  $\gamma$ -secretase processing.

5 Cotransfection with Asp2 consistently increases the ratio of A $\beta$ 42/total A $\beta$ . Values tabulated are A $\beta$  peptide pg/ml.

		pcDNA Cotransfection			Asp2 Cotransfection		
		A $\beta$ 40	A $\beta$ 42	A $\beta$ 42/Tot al	A $\beta$ 40	A $\beta$ 42	A $\beta$ 42/Tot al
10	APP	192 $\pm$ 1 8	<4	<2%	188 $\pm$ 40	8 $\pm$ 10	3.9%
	APP-VF	118 $\pm$ 1 5	15 $\pm$ 19	11.5%	85 $\pm$ 7	24 $\pm$ 12	22.4%
15	APP-KK	352 $\pm$ 2 4	21 $\pm$ 6	5.5%	1062 $\pm$ 101	226 $\pm$ 4 9	17.5%
	APP-VF-K K	230 $\pm$ 3 1	88 $\pm$ 24	27.7%	491 $\pm$ 35	355 $\pm$ 3 6	42%
20							

## Example 9

### Bacterial expression of human Asp2(a)

#### *Expression of recombinant Hu-Asp2(a) in E. coli.*

5 Hu-Asp2(a) can be expressed in *E. coli* after addition of N-terminal sequences  
such as a T7 tag (SEQ ID No. 21 and No. 22) or a T7 tag followed by a caspase 8  
leader sequence (SEQ ID No. 23 and No. 24). Alternatively, reduction of the GC  
content of the 5' sequence by site directed mutagenesis can be used to increase the  
yield of Hu-Asp2 (SEQ ID No. 25 and No. 26). In addition, Asp2(a) can be  
engineered with a proteolytic cleavage site (SEQ ID No. 27 and No. 28). To produce  
10 a soluble protein after expression and refolding, deletion of the transmembrane  
domain and cytoplasmic tail, or deletion of the membrane proximal region,  
transmembrane domain, and cytoplasmic tail is preferred. Any materials (vectors,  
host cells, etc.) and methods described herein to express Hu-Asp2(a) should in  
principle be equally effective for expression of Hu-Asp2(b).

#### 15 *Methods*

PCR with primers containing appropriate linker sequences was used to  
assemble fusions of Asp2(a) coding sequence with N-terminal sequence modifications  
including a T7 tag (SEQ ID Nos. 21 and 22) or a T7-caspase 8 leader (SEQ ID Nos.  
23 and 24). These constructs were cloned into the expression vector pet23a(+)  
20 [Novagen] in which a T7 promoter directs expression of a T7 tag preceding a  
sequence of multiple cloning sites. To clone Hu-Asp2 sequences behind the T7 leader  
of pet23a+, the following oligonucleotides were used for amplification of the selected  
Hu-Asp2(a) sequence: #553=GTGGATCCACCCAGCACGGCATCCGGCTG (SEQ  
ID No. 35), #554=GAAAGCTTTCATGACTCATCTGTCTGTGGAATGTTG (SEQ  
25 ID No. 36) which placed BamHI and HindIII sites flanking the 5' and 3' ends of the  
insert, respectively. The Asp2(a) sequence was amplified from the full length Asp2(a)  
cDNA cloned into pcDNA3.1 using the Advantage-GC cDNA PCR [Clontech]  
following the manufacturer's supplied protocol using annealing & extension at 68°C in  
a two-step PCR cycle for 25 cycles. The insert and vector were cut with BamHI and  
30 HindIII, purified by electrophoresis through an agarose gel, then ligated using the

Rapid DNA Ligation kit [Boehringer Mannheim]. The ligation reaction was used to transform the *E. coli* strain JM109 (Promega) and colonies were picked for the purification of plasmid (Qiagen, Qiaprep minispin) and DNA sequence analysis. For inducible expression using induction with isopropyl b-D-thiogalactopyranoside (IPTG), the expression vector was transferred into *E. coli* strain BL21 (Statagene). Bacterial cultures were grown in LB broth in the presence of ampicillin at 100 µg/ml, and induced in log phase growth at an OD600 of 0.6-1.0 with 1 mM IPTG for 4 hour at 37°C. The cell pellet was harvested by centrifugation.

To clone Hu-Asp2 sequences behind the T7 tag and caspase leader (SEQ ID Nos. 23 and 24), the construct created above containing the T7-Hu-Asp2 sequence (SEQ ID Nos. 21 and 22) was opened at the BamH1 site, and then the phosphorylated caspase 8 leader oligonucleotides #559=GATCGATGACTATCTCTGACTCTCCGCGTGAACAGGACG (SEQ ID No. 37), #560=GATCCGTCCTGTTCACGCGGAGAGTCAGAGATAGTCATC (SEQ ID No. 38) were annealed and ligated to the vector DNA. The 5' overhang for each set of oligonucleotides was designed such that it allowed ligation into the BamHI site but not subsequent digestion with BamHI. The ligation reaction was transformed into JM109 as above for analysis of protein expression after transfer to *E. coli* strain BL21.

In order to reduce the GC content of the 5' terminus of asp2(a), a pair of antiparallel oligos were designed to change degenerate codon bases in 15 amino acid positions from G/C to A/T (SEQ ID Nos. 25 and 26). The new nucleotide sequence at the 5' end of asp2 did not change the encoded amino acid and was chosen to optimize *E. Coli* expression. The sequence of the sense linker is 5'

CGGCATCCGGCTGCCCCCTGCGTAGCGGTCTGGGTGGTGCTCCACTGGGTCT  
GCGTCTGCCCCGGGAGACCGACGAA G 3' (SEQ ID No. 39). The sequence of  
the antisense linker is : 5'

CTTCGTCGGTCTCCCGGGGCAGACGCAGACCCAGTGGAGCACCACCCAGACCGCTACGCAGGGGCAGCCGGATGCCG 3' (SEQ ID No. 40). After annealing the phosphorylated linkers together in 0.1 M NaCl-10 mM Tris, pH 7.4 they were ligated into unique Cla I and Sma I sites in Hu-Asp2 in the vector pTAC. For

inducible expression using induction with isopropyl b-D-thiogalactopyranoside (IPTG), bacterial cultures were grown in LB broth in the presence of ampicillin at 100 ug/ml, and induced in log phase growth at an OD600 of 0.6-1.0 with 1 mM IPTG for 4 hour at 37°C. The cell pellet was harvested by centrifugation.

5 To create a vector in which the leader sequences can be removed by limited proteolysis with caspase 8 such that this liberates a Hu-Asp2 polypeptide beginning with the N-terminal sequence GSFV (SEQ ID Nos. 27 and 28), the following procedure was followed. Two phosphorylated oligonucleotides containing the caspase 8 cleavage site IETD, #571=5'

10 GATCGATGACTATCTCTGACTCTCCGCTGGACTCTGGTATCGAAACCGACG (SEQ ID No. 41) and #572=

GATCCGTCGGTTTCGATACCAGAGTCCAGCGGAGAGTCAGAGATAGTCAT C (SEQ ID No. 42) were annealed and ligated into pET23a+ that had been opened with BamHI. After transformation into JM109, the purified vector DNA was  
15 recovered and orientation of the insert was confirmed by DNA sequence analysis.

The following oligonucleotides were used for amplification of the selected Hu-Asp2(a) sequence: #573=5'AAGGATCCTTTGTGGAGATGGTGGACAACCTG, (SEQ ID No. 43) #554=GAAAGCTTTCATGACTCATCTGTCTGTGGAATGTTG (SEQ ID No. 44) which placed BamHI and HindIII sites flanking the 5' and 3' ends of  
20 the insert, respectively. The Hu-Asp2(a) sequence was amplified from the full length Hu-Asp2(a) cDNA cloned into pcDNA3.1 using the Advantage-GC cDNA PCR [Clontech] following the manufacturer's supplied protocol using annealing & extension at 68°C in a two-step PCR cycle for 25 cycles. The insert and vector were cut with BamHI and HindIII, purified by electrophoresis through an agarose gel, then  
25 ligated using the Rapid DNA Ligation kit [Boehringer Mannheim]. The ligation reaction was used to transform the *E. coli* strain JM109 [Promega] and colonies were picked for the purification of plasmid (Qiagen, Qiaprep minispin) and DNA sequence analysis. For inducible expression using induction with isopropyl b-D-thiogalactopyranoside (IPTG), the expression vector was transferred into *E. coli*  
30 strain BL21 (Statagene). Bacterial cultures were grown in LB broth in the presence of





Summary: Lysis of bacterial pellet in KCl solution, followed by centrifugation in a GSA rotor was used to initially prepare the pellet. The same solution was then used an additional three times for resuspension/homogenization. A final water wash/homogenization was then performed to remove excess KCl and EDTA.

## 5 Solubilization of Recombinant Hu-Asp2(a):

A ratio of 9-10ml/gram of pellet was utilized for solubilizing the rHuAsp2L from the pellet previously described. 17.75g of pellet was thawed, and 150ml of 8M guanidine HCl, 5mM  $\beta$ ME, 0.1% DEA, was added. 3M Tris was used to titrate the pH to 8.6. The pellet was initially resuspended into the guanidine solution using a 20 mm tissue homogenizer probe at 1000 rpm. The mixture was then stirred at 4°C for 1 hour prior to centrifugation at 12,500 rpm for 1 hour in GSA rotor. The resultant supernatant was then centrifuged for 30 minutes at 40,000 x g in an SS-34 rotor. The final supernatant was then stored at -20°C, except for 50 ml.

15 *Immobilized Nickel Affinity Chromatography of Solubilized Recombinant Hu-Asp2(a):*

The following solutions were utilized:

- A) 6M Guanidine HCl, 0.1M NaP, pH 8.0, 0.01M Tris, 5mM  $\beta$ ME, 0.5mM Imidazole
- 20 A') 6M Urea, 20mM NaP, pH 6.80, 50mM NaCl
- B') 6M Urea, 20mM NaP, pH 6.20, 50mM NaCl, 12mM Imidazole
- C') 6M Urea, 20mM NaP, pH 6.80, 50mM NaCl, 300mM Imidazole

Note: Buffers A' and C' were mixed at the appropriate ratios to give intermediate concentrations of Imidazole.

25 The 50ml of solubilized material was combined with 50ml of buffer A prior to adding to 100-125ml Qiagen Ni-NTA SuperFlow (pre-equilibrated with buffer A) in a 5 x 10cm Bio-Rad econo column. This was shaken gently overnight at 4°C in the cold room.

### Chromatography Steps:

30 Drained the resultant flow through.

Washed with 50ml buffer A (collecting into flow through fraction)

Washed with 250ml buffer A (wash 1)

Washed with 250ml buffer A (wash 2)  
Washed with 250ml buffer A'  
Washed with 250ml buffer B'  
Washed with 250ml buffer A'  
5 Eluted with 250ml 75mM Imidazole  
Eluted with 250ml 150mM Imidazole (150-1)  
Eluted with 250ml 150mM Imidazole (150-2)  
Eluted with 250ml 300mM Imidazole (300-1)  
Eluted with 250ml 300mM Imidazole (300-2)  
10 Eluted with 250ml 300mM Imidazole (300-3)

*Chromatography Results:*

The Hu-Asp(a) eluted at 75mM Imidazole through 300mM Imidazole. The 75mM fraction, as well as the first 150mM Imidazole (150-1) fraction contained  
15 contaminating proteins as visualized on Coomassie Blue stained gels. Therefore, fractions 150-2 and 300-1 will be utilized for refolding experiments since they contained the greatest amount of protein as visualized on a Coomassie Blue stained gel.

*Refolding Experiments of Recombinant Hu-Asp2(a):*

20 *Experiment 1:*

Forty ml of 150-2 was spiked with 1M DTT, 3M Tris, pH 7.4 and DEA to a final concentration of 6mM, 50mM, and 0.1% respectively. This was diluted suddenly (while stirring) with 200ml of (4°C) cold 20mM NaP, pH 6.8, 150mM NaCl. This dilution gave a final Urea concentration of 1M. This solution remained clear, even if  
25 allowed to set open to the air at room temperature (RT) or at 4°C .

After setting open to the air for 4-5 hours at 4°C, this solution was then dialyzed overnight against 20mM NaP, pH 7.4, 150mM NaCl, 20% glycerol. This method effectively removes the urea in the solution without precipitation of the protein.

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**SECRET**

### 5 Experiment 3:

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### Expression of Hu-Asp2 and Derivatives in Insect Cells

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### *Expression by baculovirus infection.*

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contains the natural translation termination codon in the Hu-Asp2 sequence. PCR amplification of the pcDNA3.1(hygro)/Hu-Asp2(a) template was used to prepare two derivatives of Hu-Asp2(a) or Hu-Asp(b) that delete the C-terminal transmembrane domain (SEQ ID Nos. 29-30 and 50-51, respectively) or delete the transmembrane domain and introduce a hexa-histidine tag at the C-terminus (SEQ ID Nos. 31-32 and 52-53) respectively, were also engineered using PCR. The same 5'-sense oligonucleotide primer described above was paired with either a 3'-antisense primer that (1) introduced a translation termination codon after codon 453 (SEQ ID No. 3) or (2) incorporated a hexa-histidine tag followed by a translation termination codon in the PCR using pcDNA3.1(hygro)/Hu-Asp-2(a) as the template. In all cases, the PCR reactions were performed amplified for 15 cycles using *Pwo*I DNA polymerase (Boehringer-Mannheim) as outlined by the supplier. The reaction products were digested to completion with *Bam*HI and *Not*I and ligated to *Bam*HI and *Not*I digested baculovirus transfer vector pVL1393 (Invitrogen). A portion of the ligations was used to transform competent *E. coli* DH5\_ cells followed by antibiotic selection on LB-Amp. Plasmid DNA was prepared by standard alkaline lysis and banding in CsCl to yield the baculovirus transfer vectors pVL1393/Asp2(a), pVL1393/Asp2(a) $\Delta$ TM and pVL1393/Asp2(a) $\Delta$ TM(His)<sub>6</sub>. Creation of recombinant baculoviruses and infection of sf9 insect cells was performed using standard methods.

#### Expression by transfection

Transient and stable expression of Hu-Asp2(a) $\Delta$ TM and Hu-Asp2(a) $\Delta$ TM(His)<sub>6</sub> in High 5 insect cells was performed using the insect expression vector pIZ/V5-His. The DNA inserts from the expression plasmids vectors pVL1393/Asp2(a), pVL1393/Asp2(a) $\Delta$ TM and pVL1393/Asp2(a) $\Delta$ TM(His)<sub>6</sub> were excised by double digestion with *Bam*HI and *Not*I and subcloned into *Bam*HI and *Not*I digested pIZ/V5-His using standard methods. The resulting expression plasmids, referred to as pIZ/Hu-Asp2 $\Delta$ TM and pIZ/Hu-Asp2 $\Delta$ TM(His)<sub>6</sub>, were prepared as described above.

For transfection, High 5 insect cells were cultured in High Five serum free medium supplemented with 10  $\mu$ g/ml gentamycin at 27°C in sealed flasks.

Transfections were performed using High five cells, High five serum free media supplemented with 10 µg/ml gentamycin, and InsectinPlus liposomes (Invitrogen, Carlsbad, CA) using standard methods.

For large scale transient transfections,  $1.2 \times 10^7$  high five cells were plated in a 150 mm tissue culture dish and allowed to attach at room temperature for 15-30 minutes. During the attachment time the DNA/ liposome mixture was prepared by mixing 6 ml of serum free media, 60 µg Hu-Asp2(a)ΔTM/pIZ (+/- His) DNA and 120 µl of Insectin Plus and incubating at room temperature for 15 minutes. The plating media was removed from the dish of cells and replaced with the DNA/liposome mixture for 4 hours at room temperature with constant rocking at 2 rpm. An additional 6 ml of media was added to the dish prior to incubation for 4 days at 27 °C in a humid incubator. Four days post transfection the media was harvested, clarified by centrifugation at 500 x g, assayed for Hu-Asp2(a) expression by Western blotting. For stable expression, the cells were treated with 50 µg/ml Zeocin and the surviving pool used to prepared clonal cells by limiting dilution followed by analysis of the expression level as noted above.

*Purification of Hu-Asp2(a)ΔTM and Hu-Asp2(a)ΔTM(His)<sub>6</sub>*

Removal of the transmembrane segment from Hu-Asp2(a) resulted in the secretion of the polypeptide into the culture medium. Following protein production by either baculovirus infection or transfection, the conditioned medium was harvested, clarified by centrifugation, and dialyzed against Tris-HCl (pH 8.0). This material was then purified by successive chromatography by anion exchange (Tris-HCl, pH 8.0) followed by cation exchange chromatography (Acetate buffer at pH 4.5) using NaCl gradients. The elution profile was monitored by (1) Western blot analysis and (2) by activity assay using the peptide substrate described in Example 12. For the Hu-Asp2(a)ΔTM(His)<sub>6</sub>, the conditioned medium was dialyzed against Tris buffer (pH 8.0) and purified by sequential chromatography on IMAC resin followed by anion exchange chromatography.

Amino-terminal sequence analysis of the purified Hu-Asp2(a) $\Delta$ TM(His)<sub>6</sub> protein revealed that the signal peptide had been cleaved [TQHGI~~R~~LPLR, corresponding to SEQ ID NO: 32, residues 22-3].

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### Example 11

#### Expression of Hu-Asp2(a) and Hu-Asp(b) in CHO cells

The materials (vectors, host cells, etc.) and methods described herein for expression of Hu-Asp2(a) are intended to be equally applicable for expression of Hu-Asp2(b).

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#### *Heterologous expression of Hu-Asp-2(a) in CHO-K1 cells*

The entire coding sequence of Hu-Asp2(a) was cloned into the mammalian expression vector pcDNA3.1(+)~~Hygro~~ (Invitrogen, Carlsbad, CA) which contains the CMV immediate early promoter and bGH polyadenylation signal to drive over expression. The expression plasmid, pcDNA3.1(+)~~Hygro~~/Hu-Asp2(a), was prepared by alkaline lysis and banding in CsCl and completely sequenced on both strands to verify the integrity of the coding sequence.

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Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from the ATCC. The cells were maintained in monolayer cultures in  $\alpha$ -MEM containing 10% FCS at 37°C in 5% CO<sub>2</sub>. Two 100 mm dishes of CHO-K1 cells (60% confluent) were transfected with pcDNA3.1(+)~~Hygro~~ alone (mock) or pcDNA3.1(+)~~Hygro~~/Hu-Asp2(a) or pcDNA3.1(+)~~Hygro~~/Hu-Asp2(b) using the cationic liposome DOTAP as recommended by the supplier (Roche, Indianapolis, IN). The cells were treated with the plasmid DNA/liposome mixtures for 15 hours and then the medium replaced with growth medium containing 500 Units/ml hygromycin B. In the case of pcDNA3.1(+)~~Hygro~~/Hu-Asp2(a) or (b) transfected CHO-K1 cells, individual hygromycin B-resistant cells were cloned by limiting dilution. Following clonal expansion of the individual cell lines, expression of Hu-Asp2(a) or Hu-Asp2(b) protein was assessed by Western blot analysis using a polyclonal rabbit antiserum raised against recombinant Hu-Asp2 prepared by expression in *E. coli*. Near confluent dishes of each cell line were harvested by scraping into PBS and the cells

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were pooled and dialyzed against 25 mM NaOAc (pH 4.5)/50 mM  $\beta$ -octylglucoside. Following dialysis, precipitated material was removed by centrifugation and the soluble material chromatographed on a MonoS cation exchange column that was previously equilibrated in 25 mM NaOAc (pH 4.5)/ 50 mM  $\beta$ -octylglucoside. The column was eluted using a linear gradient of increasing NaCl concentration (0-1.0 M over 30 minutes) and individual fractions assayed by Western blot analysis and for  $\beta$ -secretase activity. Fractions containing both Hu-Asp2 immunoreactivity and  $\beta$ -secretase activity were combined and determined to be >95% pure by SDS-PAGE/Coomassie Blue staining.

The same methods were used to express and purify Hu-Asp2(b).

### Example 12

#### Assay of Hu-Asp2 $\beta$ -secretase activity using peptide substrates

##### *$\beta$ -secretase assay*

Recombinant human Asp2(a) prepared in CHO cells and purified as described in Example 11 was used to assay Asp2(a) proteolytic activity directly. Activity assays for Asp2(a) were performed using synthetic peptide substrates containing either the wild-type APP  $\beta$ -secretase site (SEVKM↓DAEFR; SEQ ID NO: 64), the Swedish KM-NL mutation (SEVNL↓DAEFR; SEQ ID NO: 63), or the A $\beta$ 40 and 42  $\gamma$ -secretase sites (RRGGVV↓IA↓TVIVGER; SEQ ID NO: 65). Reactions were performed in 50 mM 2-[N-morpholino]ethane-sulfonate ("Na-MES," pH 5.5) containing 1%  $\beta$ -octylglucoside, 70 mM peptide substrate, and recombinant Asp2(a) (1-5  $\mu$ g protein) for various times at 37°C. The reaction products were quantified by RP-HPLC using a linear gradient from 0-70 B over 30 minutes (A=0.1% TFA in water, B=0.1%TFA/10%water/90%AcCN). The elution profile was monitored by absorbance at 214 nm. In preliminary experiments, the two product peaks which eluted before the intact peptide substrate, were confirmed to have the sequence DAEFR (SEQ ID NO: 72)and SEVNL (SEQ ID NO: 73) using both Edman sequencing and MADLI-TOF mass spectrometry. Percent hydrolysis of the peptide substrate was calculated by comparing the integrated peak areas for the two product



peptides and the starting material derived from the absorbance at 214 nm. The sequence of cleavage/hydrolysis products was confirmed using Edman sequencing and MADLI-TOF mass spectrometry.

The behavior of purified Asp2(a) in the proteolysis assays was consistent with the prior anti-sense studies which indicated that Asp2(a) possesses  $\beta$ -secretase activity. Maximal proteolysis was seen with the Swedigh  $\beta$ -secretase peptide, which, after 6 hours, was about 10-fold higher than wild type APP.

The specificity of the protease cleavage reaction was determined by performing the  $\beta$ -secretase assay in the presence of 8  $\mu$ M pepstatin A and the presence of a cocktail of protease inhibitors (10  $\mu$ M leupeptin, 10  $\mu$ M E64, and 5 mM EDTA). Proteolytic activity was insensitive to both the pepstatin and the cocktail, which are inhibitors of cathepsin D (and other aspartyl proteases), serine proteases, cysteinyl proteases, and metalloproteases, respectively.

15 Hu-Asp2(b) when similarly expressed in CHO cells and purified using identical conditions for extraction with  $\beta$ -octylglucoside and sequential chromatography over Mono Q and Mono S also cleaves the Swedish  $\beta$ -secretase peptide in proteolysis assays using identical assay conditions.

Collectively, this data establishes that both forms of Asp2 (Hu-Asp2(a) and Hu-Asp2(b)) act directly in cell-free assays to cleave synthetic APP peptides at the  $\beta$ -secretase site, and that the rate of cleavage is greatly increased by the Swedish KM-NL mutation that is associated with Alzheimer's disease.

An alternative  $\beta$ -secretase assay utilizes internally quenched fluorescent substrates to monitor enzyme activity using fluorescence spectroscopy in a single sample or multiwell format. Each reaction contained 50 mM Na-MES (pH 5.5), peptide substrate MCA-EVKMDAEF[K-DNP] (SEQ ID NO: 71; BioSource International) (50  $\mu$ M) and purified Hu-Asp-2 enzyme. These components were equilibrated to 37  $^{\circ}$ C for various times and the reaction initiated by addition of substrate. Excitation was performed at 330 nm and the reaction kinetics were monitored by measuring the fluorescence emission at 390 nm. To detect compounds that modulate Hu-Asp-2 activity, the test compounds were added during the

preincubation phase of the reaction and the kinetics of the reaction monitored as described above. Activators are scored as compounds that increase the rate of appearance of fluorescence while inhibitors decrease the rate of appearance of fluorescence.

5           It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the invention. The entire disclosure of all publications cited herein are hereby incorporated by reference.

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